Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 83 JULY, 1953 No. 3

SECTION MEETINGS

DISTRICT OF COLUMBIA
Washington D. C. June 4, 1953
SOUTHWESTERN
University of Oklahoma Medical School May 8-9, 1953

Plasma Coagulation Factors in Acute Pancreatitis.* (20377)

GEORGE Y. SHINOWARA, JOHN H. WAITE,† AND RICHARD G. SALEEBY.†

From the Departments of Pathology and of Surgery, College of Medicine, Ohio State University, Columbus.

Since thrombosis has been associated with pancreatic diseases(1), particularly with carcinoma of the pancreas(2) some investigations on the blood coagulation mechanism(3-6) in this group of diseases have been made. For some time in this laboratory coagulation components have been determined quantitatively on blood fractions(7). By this isolation procedure it has been possible to estimate specifically the concentrations of prothrombin, fibrinogen, thromboplastic cell component and thromboplastic plasma component. This communication contains data demonstrating significant elevation of thromboplastic plasma component in patients with acute pancreatitis and in dogs with experimental pancreatitis. Serum amylase, prothrombin and fibrinogen results are also reported.

Methods. About 8 ml of blood were obtained by venipuncture and allowed to clot. The syringe was replaced by one containing 1.5 ml 4% sodium citrate solution and approximately 15 ml of blood were collected. The citrated blood was centrifuged in calibrated 15 ml graduated tubes at 1°C, 1,650 g, for 30 minutes. Fibrinogen, thromboplastic plasma component and prothrombin were determined on the protein fractions obtained from 4 ml of plasma by a low temperature alcohol procedure(7). Serum amylase was determined by Somogyi's saccharogenic technic(8).

Results. Normal values. Serum amylase levels below 60 and above 200 are considered abnormal in human beings(9). Normal plasma fibrinogen and prothrombin concentrations are respectively 0.26, S.D. ± 0.05% and 81.8, S.D. ± 14.4 units/ml(7). In the present study the mean thromboplastic plasma component (TPC) concentration in 31 normal men and women was found to be 60.4, S.D. ± 34.8 units/ml. In 13 healthy untreated adult dogs weighing between 9 and 18 kg the means and ranges were: amylase, 1309 (695-1970) units/100 ml; TPC, 1112 (64-3848) units/ml; fibrinogen, 0.329% (0.139-0.585%); and prothrombin, 39.3 (24.3-58.8) units/ml. Therefore in such animals the average amy-

---

* Supported in part by grants from the National Institutes of Health and the Comly Fund.

† In partial fulfillment of the requirements for Master of Medical Science degree in Surgery; Dr. Waite, member of Public Health Service, National Institutes of Health.

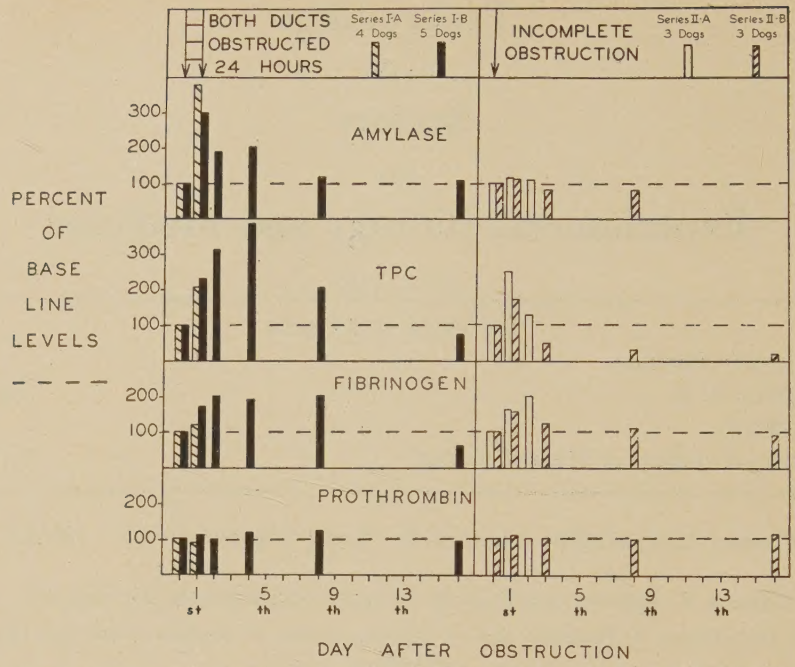


FIG. 1. Changes in serum amylase and plasma coagulation factors in dogs following obstruction of pancreatic ducts.

lase and TPC levels are about 10 and 20 times higher respectively than in human beings. The prothrombin level in dogs is about one-half that in human beings.

Experimental pancreatitis. Sublethal attacks of pancreatitis were produced in dogs by a technic previously described in detail(10). The major and minor pancreatic ducts were each encircled with loops of nylon tubing 1.5 mm in diameter. After a 3 weeks recovery period from the effects of surgery, blood specimens were obtained for baseline values (zero day, Fig. 1). Then, in the dogs of Series I-A and I-B, both ducts were obstructed by tying the ends of the nylon loops under tension. After 24 hours, the nylon loops were released and blood specimens were immediately obtained: The results thus found are shown as 1st day values in the left half of Fig. 1. In all 9 dogs the amylase level was 3.3 (range, 2-5) times greater on the 1st day after 24 hours duct obstruction. The values approached baseline levels by the 8th day. The TPC level in all 9 dogs was 2.2 (range 2-4) times greater on the 1st day. The peak was reached on the 4th day, when the TPC level was 3.7 (range 2-8) times greater than the baseline, and was still elevated on the 8th day. It was slightly below baseline level on the 16th day. The fibrinogen level increased to a 2-fold level in about the 2nd day after obstruction and remained at this level on the 8th day. The prothrombin level did not change significantly. In the dogs of Series I-A and I-B, which were sacrificed, autopsy showed gross and histologic evidence of pancreatitis, characterized by fat necrosis, parenchymatous degeneration and interstitial inflammatory infiltration.

In the right half of Fig. 1 are the results obtained on 6 dogs (Series II-A and II-B) in which only one nylon loop was tied or in which autopsy disclosed that one or both nylon loops had broken. Under these conditions the serum amylase values did not deviate more than one-fourth of the baseline levels. The TPC and fibrinogen levels changed but not to the degree in respect to extent and duration demonstrated in the dogs whose ducts were completely obstructed for 24 hours. Moreover, the TPC peak was reached on the 1st day and by the 2nd day was almost back

TABLE I. Levels of Serum Amylase and of Plasma Coagulation Factors Associated with 6 Attacks of Acute Interstitial Pancreatitis in 5 Patients.

Case No.	Age, sex	Dates	Serum amylase, Somogyi, units/100 ml	Thromboplastic plasma component: TPC, units/ml	Fibrinogen, %	Prothrombin, units/ml
1283	78 ♂	9/17	744	492	.431	46.7
1347	65 ♂	1/13	498	—	—	—
		1/17	67	1302	.497	62.3
1366	39 ♂	2/25	704	112	.485	81.3
		2/26	304	83	.528	74.3
		2/27	93	137	.596	76.5
		3/2	62	311	.728	78.2
		3/3	77	299	.658	79.1
		3/4	94	279	.692	83.0
1325	70 ♀	12/15	1119	—	—	—
		12/18	406	1321	.615	64.4
1334	57 ♀	1/21	1449	—	—	—
		1/22	183	585	.316	83.8
		1/23	136	178	.288	78.1
		1/26	133	158	.280	80.8
		1/28	1107	382	.177	76.1
		1/28*	667	315	.217	77.9
		1/29	307	565	.452	71.4
		1/30	82	1123	.697	76.9
		2/2	67	549	.626	76.8
		2/5	98	145	.421	89.5
		2/5 *	59	143	.419	80.0
		2/6 †	92	238	.412	78.4

* Specimens collected 4 p.m.; all others between 8 and 9 a.m.
† 24 hr after a cholangiogram.

to the baseline level: The 4th, 8th and 16th day levels were low.

Pancreatitis in patients. The levels of plasma coagulation factors in 5 patients with acute interstitial pancreatitis and elevated serum amylase are presented in Table I. Following all 6 attacks the TPC values were 5 to 22 times the normal mean. The peak in the TPC elevation occurred several days after that of the amylase level. The TPC level remained high for over a week. There was slight to moderate elevation of plasma fibrinogen. All prothrombin results were well within the normal range except for a moderate diminution in Case 1283.

In addition 6 cases were studied on whom the diagnosis of chronic pancreatitis had been made. The investigations and the ranges of results were: amylase, 24-125 units; TPC, 14-77 units; fibrinogen, 0.20-0.57%; and prothrombin, 24-71 units.

Discussion. In a variety of pathologic conditions studied thus far in this laboratory, nonfatal acute pancreatitis is the first in which consistently marked TPC elevations have been demonstrated. A definite deficiency of this factor in hemophilic blood plasma was previously reported(11). TPC is an essential factor in the blood coagulation mechanism because together with thromboplastic cell component, a lipoprotein of blood cells, it quantitatively converts prothrombin to thrombin(7). In view of the foregoing, an excess of TPC could be interpreted as increasing the coagulability of blood. However, the importance of this finding in respect to thrombosis associated with pancreatic diseases can be determined only by further investigations.

Summary. Following 6 attacks of acute interstitial pancreatitis in 5 patients and the obstruction of both pancreatic ducts in 9 dogs, the thromboplastic plasma component showed a consistently marked elevation. The significance of this observation is discussed. The increase in plasma fibrinogen concentration in acute pancreatitis was confirmed. Prothrombin did not vary significantly. In addition the levels of plasma coagulation fac-

tors in 6 patients with chronic pancreatitis, in 6 dogs with incomplete obstruction of the pancreatic ducts and in 13 normal untreated dogs are reported. The coagulation factors were determined quantitatively on plasma fractions obtained by a low temperature procedure. Amylase determinations were done on all specimens.

The authors are grateful to Mrs. Dolores M. Johnston, Miss Marilyn J. Herritt, Miss Elizabeth P. Lorey and Mrs. Anne W. Tatum for the determination of the coagulation factors; to Miss Rachel B. Earp, Miss Donna F. Bitzel, Miss Margaret A. Delaney and Miss Martha I. Walters for the amylase determinations; and to Dr. Robert M. Zollinger for making his patients available for this study.

---

1. Smyth, C. J., *Arch. Path.*, 1940, v30, 651.
2. Thompson, C. M., and Rodgers, L. R., *Am. J. Med. Sci.*, 1952, v223, 469.
3. Ferrari, R., and Cortese, F., *Arch. di Fisiol.*, 1932, v31, 274.
4. Lasher, E. P., and McCabe, H. M., *Arch. Surg.*, 1950, v60, 164.
5. Innerfield, I., Angrist, A., and Benjamin, J. W., *Gastroenterol.*, 1951, v19, 843.
6. McFarlane, J. C. W., *Lancet*, 1952, v263, 311.
7. Shinowara, G. Y., *J. Lab. and Clin. Med.*, 1951, v38, 11.
8. Somogyi, M., *J. Biol. Chem.*, 1938, v125, 399.
9. ———, *Arch. Int. Med.*, 1941, v67, 665.
10. Waite, J. H., *Surgical Forum*, in press, 1952.
11. Shinowara, G. Y., *J. Lab. and Clin. Med.*, 1951, v38, 23.

Received April 10, 1953. P.S.E.B.M., 1953, v83.

A Simple Technic for Demonstrating Plaque Formation with Virus of Vaccinia.* (20378)

WILBUR FISKE NOYES (Introduced by Alice E. Moore)

From the Virus Section, Sloan-Kettering Institute for Cancer Research, Memorial Cancer Center, New York.

A technic for the demonstration of focal necrotic areas (plaques) by animal viruses would provide a more accurate means of titration. It would also be a valuable tool for studying their growth characteristics and genetic properties. In addition, it might be helpful in isolating mutant viruses and in studying antiviral substances. There has been publication on such a technic. Gey and Bang(1) stated briefly that they had been able to produce focal necrosis in static slide cultures of a rat sarcoma with the virus of Eastern equine encephalomyelitis. Recently Dulbecco(2) reported in detail on plaque formation in chick embryo tissue cultures by the viruses of Western equine encephalomyelitis and Newcastle Disease. The present paper describes a simple, readily reproducible technic whereby plaque formation has been consistently obtained with the virus of vaccinia.

Materials and methods. The cell suspension. Five, 11-day-old chick embryos were decapitated, washed twice with Gey's saline (GS) and pressed into 15 ml of GS through a wire mesh strainer, having 15 strands per cm. This material was centrifuged at 110 x G for 30 seconds and the resulting supernatant was discarded. The sediment was resuspended, pipetted rapidly 20 times with a 10 ml pipette and centrifuged at 110 x G for 15 seconds. The supernatant was then removed and allowed to stand for 20 minutes, during which time the large particles sedimented into the bottom 1 ml. This was discarded and the resulting preparation consisted, for the most part, of a suspension of single cells. The cell concentration of the suspension was then determined in a hemocytometer and an amount added to the nutrient fluid which resulted in a final cell concentration of 8 x 10^6 cells per ml. This usually required about 10 ml of the cell

---

* This work was supported by funds from the National Cancer Institute of the U. S. Public Health Service, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

TABLE I. Relation between Number of Plaques Formed in Culture and Concentration of Vaccinia Virus. Virus sample was .1 ml except where noted.

	Virus dilution			
Exp.	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	Confluent	237	21, 21, 23	4
2	”	115	9	2
3	”	125, 112	32 (.4 ml)	—
4	”	475	32	—
5	—	—	28, 24 (.2 ml), 65 (.4 ml)	—
6	—	252 (.2 ml)	27, 24 (.2 ml)	3 (.2 ml)
Avg		198	16.5	2.5

suspension per 20 ml of nutrient fluid. *The nutrient fluid.* This was composed of 40% horse serum heated to 56° for 30 minutes; 20% embryo extract (1:1 in GS) prepared from 10-day-old embryos; and 40% GS. Fifty units of penicillin and 50 μg of streptomycin were added per ml. The initial pH of the nutrient fluid was 7.6 ± .1. *Cultures.* Cultures were set up in regular 100 x 15 mm test tubes, in 50 mm Carrel flasks, or in 50 mm Petri dishes (incubated in an atmosphere of 5% CO_2). The flask preparations had the advantage of very uniform cell sheets while the Petri dish preparations had the advantage of accessibility. Cultures were also prepared on small cover slips (5 x 20 mm) for microscopic examination of stained preparations. It was found that the cells attached themselves more firmly and formed a more homogeneous sheet when they were allowed to settle onto a glass surface which had been coated with a thin clot of chicken plasma. This consisted of .4 ml of plasma (heparin 1:50000) and .1 ml of embryo extract (1:1 in GS) for the flask and dish preparations. After the clot had hardened 3 ml of nutrient fluid containing 8 x 10^6 cells per ml were added per flask or dish and the cultures incubated overnight at 37°. After overnight incubation a fairly uniform sheet of cells had formed, and the virus sample was added at that time. *Vaccinia virus.* Three different strains were used: 1) smallpox vaccine obtained from Lederle Laboratories (Lot No. 2095-205B and Lot No. 2095-206A), 2) a calf lymph preparation that had been passed 4 times in the rabbit's testicle and twice on the chorioallantoic membrane (CAM) of the chick embryo, 3) a strain of Levaditi's neurovaccinia that had been passed 23 times in the rabbit's testicle and twice on the CAM. The latter two strains were obtained from Duran-Reynals. All virus stocks were cultured to make certain that they were free from bacteria. After removing the nutrient fluid and washing the tissue cultures with 2 ml of GS, the virus inoculum was added and the flasks incubated at 35° for one hour. A thin clot of chicken plasma was then placed over the cell sheet, since this resulted in a finer, more uniform sheet and more discrete lesions. Plaques were readily produced even without addition of this plasma clot. After the clot hardened 3 ml of nutrient fluid were added. The pH of this fluid dropped rapidly and it was necessary to change it on the third day after adding the virus. *Vital staining of the cultures.* Since it was difficult to visualize the plaques in unstained preparations, neutral red (National Aniline) in GS was added to the nutrient fluid (at a pH of 6.8-7.0) to give a final concentration of 1:5000.

Results. Plaques were consistently produced with all of the strains of vaccinia virus and were readily visible upon staining with neutral red as early as 48 hours after the addition of virus. These appeared as round, white, opaque areas of destroyed cells against a background of pinkly stained living cells. The plaques reached a maximum diameter of about 1 mm on the fifth day. Microscopic examination of fixed and stained preparations of plaques formed in coverslip cultures, showed the plaques to be composed of cell debris, surrounded by healthy fibroblast-type cells. In the cover-slip cultures, typical eosinophilic, cytoplasmic, inclusion bodies of vaccinia were seen in the cells surrounding the focal lesions after staining with hematoxylin and eosin. The plaques were always absent when the

Plaque formation by the virus of vaccinia in chick embryo tissue cultures. These cultures were stained with neutral red.

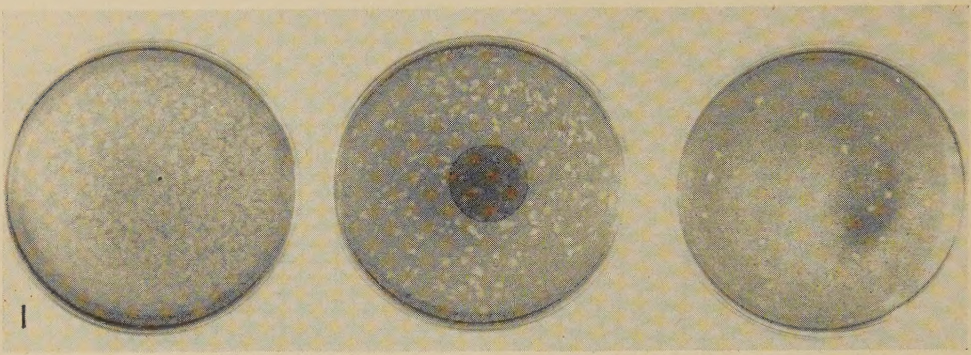
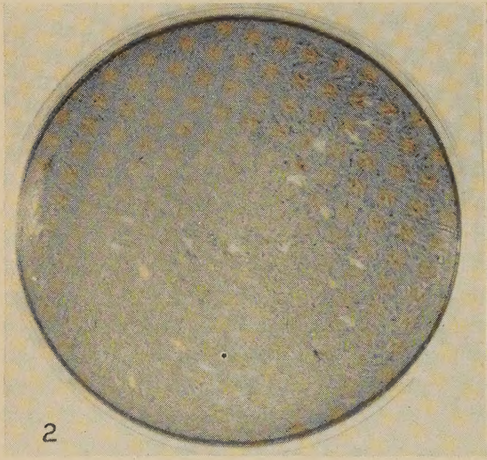


FIG. 1. Petri dish preparation showing effect of virus dilution on number of plaques. Virus dilution left to right: 10^{-3}, 10^{-4}, 10^{-5}. (Filter disc in center of middle culture was used in an unsuccessful attempt to demonstrate inhibition by an antimetabolite.)



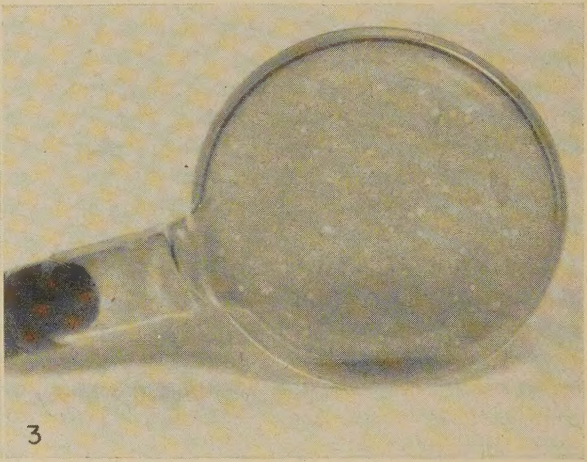


FIG. 2. Petri dish preparation without upper plasma clot showing less discrete plaques and less homogeneous cell sheet. Virus dilution: 10^{-5}.

FIG. 3. Carrel flask preparation showing uniform cell sheet and discrete lesions. Virus dilution: 10^{-5}.

virus was absent and their number increased in proportion to the amount of virus added, as illustrated in Table I. In the lower dilutions (10^{-2}, 10^{-3}) practically all the cells were destroyed, with release of the dye into solution, and in the higher dilutions (10^{-4}, 10^{-5}, 10^{-6}) discrete, separated plaques were obtained. This is illustrated in Fig. 1, 2. A comparative study with the chick embryo demonstrated that the number of pox produced on the CAM by the virus of vaccinia correlates with the number of plaques produced in culture, although the titer was almost 6-fold higher *in vivo*. For example, at 10^{-6} dilution the averages were 2.5 plaques and 14 pox. In a number of different experiments vaccinia virus was incubated with rabbit antivaccinial serum for one hour at 37°. The number of pox formed on the CAM and the number of plaques formed in culture were consequently reduced 95% (Table II). Normal serum under the same conditions had no effect on the number of plaques formed in culture. In several recent experiments the virus inoculum was added in 2 ml of GS and this was replaced with 3 ml of nutrient fluid after 24 hours. At 48 hours the cell sheet was covered with a plasma clot and 3 ml of

TABLE II. Effect of Antivaccinial Rabbit Serum on Plaque Formation in Culture and Pock Production on Chorioallantoic Membrane of the Chick Embryo. An equal amount of serum was added to virus dilution (10^{-5}) and mixture incubated at 37° for one hour.

	Exp.	Control	Antiserum	% reduction
Plaques	1	28, 24 (.2 ml), 65 (.4 ml)	1, 2 (.4 ml)	95
	3	27, 24 (.2 ml)	10 (.4 ml)*	61
Pox	1	32, 22, 58, 61 (.05 ml)	2, 1, 1, 2 (.1 ml)	96
	2	24, 42, 40, 44, 38 (.05 ml)	3, 1, 1, 3, 1, 2 (.1 ml)	95

* Serum frozen-thawed 4×.

fresh nutrient fluid added. Using this modified technic a plaque to pock ratio close to 1:1 was obtained.

Discussion and summary. A simple technic is described whereby the production of plaques in chick embryo tissue cultures has been consistently obtained with vaccinia virus. The plaques are probably due to viral activity. This conclusion is supported by the fact that 1) the plaques are absent when the virus is absent and their number increases with the concentration of the virus, 2) the number of plaques produced in tissue culture correlates with the number of pox produced on the CAM of the developing chick embryo and 3) specific antibody reduces both the number of plaques formed in tissue culture and the number of pox formed on the CAM by 95%.

1. Gey, G. O., and Bang, F. B., *Trans. New York Acad. Sci.*, 1951, v14, 15.

2. Dulbecco, R., *Proc. Nat. Acad. Sci.*, 1952, v38, 747.

Received April 27, 1953. P.S.E.B.M., 1953, v83.

Characteristic Individual Electrophoretic Patterns in Humans.* (20379)

PETER BERNFELD, VIRGINIA M. DONAHUE AND F. HOMBURGER.

From the Cancer Research and Cancer Control Unit, Departments of Surgery, of Biochemistry, and of Medicine, Tufts College Medical School, Boston, Mass.

The electrophoretic analysis of serum and plasma proteins in normal individuals and in patients with a variety of diseases has been repeatedly described(1,2). Six to 9 different protein components can generally be recognized in the plasma at pH 8.6. It is customary to characterize these protein components by their electrophoretic mobility and to determine their relative amounts by measuring the surface area of the electrophoretic patterns. From the data thus obtained and from the value for total plasma protein, determined by chemical analysis, the absolute concentrations of the plasma protein components can then be computed. Consequently, the electrophoresis of human plasma is generally considered to be a method for quantitative determination of some of the protein constituents of blood. Many attempts have been made to correlate the values obtained by electrophoresis for the various protein components with disease, age, sex, and other conditions of the blood donor. In most instances the plasma protein composition appears to be rather independent of these factors. Only in a few cases are the electrophoretic plasma protein values more or less specific for a disease, such as p.e. in multiple myeloma. In the course of studies on plasma proteins in patients from 3 separate hospitals and in a number of normal individuals, 1250 electrophoretic plasma analyses of 440 individuals have been carried out. In many cases, analyses have been performed on the same person at different time intervals, ranging from several hours to more than 2 years. Our findings on the relationship between plasma protein composition and factors such as disease, nutrition and age will be reported elsewhere.

---

* This study has been supported by an Institutional Grant of the American Cancer Society, New York City and of the Damon Runyon Memorial Fund for Cancer Research, New York.

The purpose of the present paper is to document with data our view that the careful examination of the general and detailed morphological appearance of the electrophoretic patterns yields valuable information which cannot be obtained by calculating the relative amounts or the electrophoretic mobilities of the protein components alone.

Methods. 5 to 10 ml of fresh venous blood were added to 0.3 g of crystalline sodium citrate. The dissolution of the crystals in the blood was accelerated by gentle shaking. Immediately thereafter, the red cells were separated by centrifugation for 10 minutes at 2000 r.p.m. The plasma was stored in the refrigerator at +3°C until used for electrophoresis study, but generally not for more than 2 to 3 days. If it had to be kept for longer periods, it was shell-frozen at –70°C and then stored at –15°C. Two ml of citrated plasma were dialysed in Visking sausage casings for 48 hours in the refrigerator against 500 ml of a veronal-citrate buffer of pH 8.6 and of ionic strength $\mu = 0.1$ (containing 0.06 M diethylbarbiturate and 0.008 M citrate). The dialysed plasma was then diluted with 0.7 ml of distilled water when the total protein was 7%, and the solution was made up to 7.0 ml with the above mentioned buffer. For plasma with other total protein values, 0.1 ml of distilled water were added for each 1% of total protein. The purpose of the addition of distilled water to the dialysed and diluted plasma is to accomplish the total or almost total elimination of some of the known electrophoretic anomalies, such as the delta- and epsilon-salt gradients and other asymmetries between the patterns of the ascending and descending limb of the cell(3,4). The *electrophoretic analyses* were carried out in a 2.5 ml Tiselius cell of a Perkin-Elmer model 38 apparatus. The original protein-buffer boundary was displaced 9 mm into the limb of the cell, and photographically recorded by means of the Longsworth scanning method.

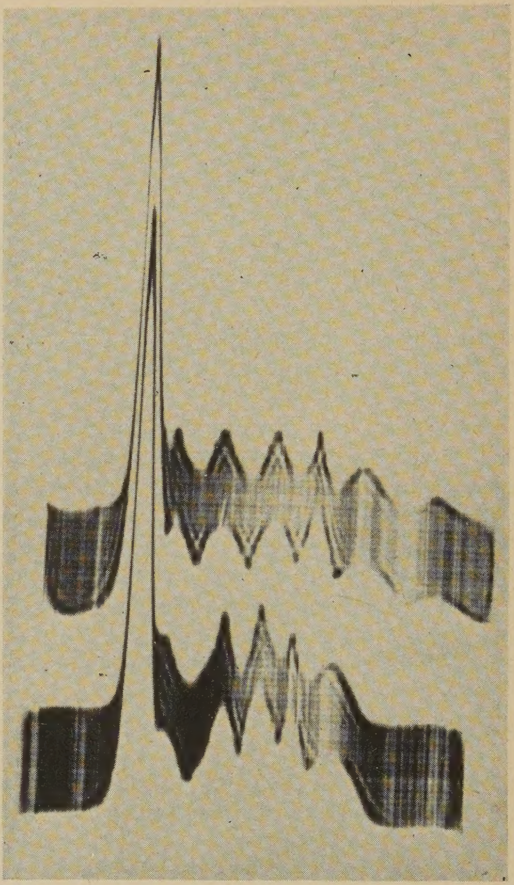


FIG. 1. Comparison of the electrophoretic patterns of two patients. Top patterns: patient S. Bottom patterns: patient M. The peaks represent from left to right: albumin, α_1-globulin, α_2-globulin, β-globulin, fibrinogen, γ-globulin, and a small remainder of the δ-salt anomaly (only in the top patterns).

The protein boundaries obtained after exposure to an electric field of 10.5 volt/cm (130 volt difference of potential between the two electrodes) for 70 minutes were photographed on the same film. The *relative amounts of the protein* components were determined according to the method of Tiselius and Kabat(5). Numerous determinations of the protein composition and of the electrophoretic mobilities from both the ascending and the descending boundaries have shown that under our experimental conditions (which are characterized in particular by the almost complete elimination of the salt anomalies through the addition of water) practically the same values are obtained from the patterns

TABLE I. Comparison of the Electrophoretic Patterns of 2 Patients.

	Patient	Albumin	α_1-glob.	α_2-glob.	β-glob.	Fibr.	γ-glob.
Relative amt of proteins*	S.	42.8	7.9	14.2	15.6	9.3	10.2
	M.	43.0	7.9	14.4	14.1	8.4	12.2
Electrophoretic mobilities†	S.	5.9	5.15	4.15	3.0	2.1	1.1
	M.	5.9	5.1	3.9	3.05	2.0	1.35

* In % of total protein. † In $cm^2\ sec^{-1}\ volt^{-1} \times 10^5$.

of either limb of the cell. As the ascending boundaries are known to yield a much better resolution of the proteins than the descending boundaries, only the patterns from the ascending boundaries are used in routine calculations of the protein composition, and all patterns reproduced in this paper are ascending boundaries.

Patients. S. (Fig. 1, Table I), 39 yr. ♀ with chronic myelogenic leukemia. M. (Fig. 1, Table I), 75 yr. ♀ with rheumatoid arthritis and arteriosclerotic heart disease. E. (Fig. 2, Table II), 47 yr. ♀ with rheumatoid arthritis. A.S. (Fig. 3, Table II), 37 yr. ♀ with cancer of breast. B.M. (Fig. 4), 49 yr. ♂ with hemolytic anemia. D. (Fig. 5), 47 yr. ♂ with rheumatoid arthritis.

Results. The general characteristics of the appearance of electrophoretic patterns. The following example illustrates the importance of thorough examination of the shape, the symmetry or asymmetry, and the width of each protein gradient, the distance between adjacent peaks and their tendency to separate from or to adhere to each other. In Fig. 1 the patterns of 2 patients are superimposed one on top of the other. The relative amounts of the plasma protein components and their electrophoretic mobilities, obtained from these patterns, are given in Table I.

It is obvious that these 2 patients yield electrophoretic patterns of quite different appearance, although there is a remarkable similarity between the relative amounts of their albumin, their α_1-globulin and their α_2-globulin. The difference in the mobilities of the α_2-globulins is by no means the only one found between these 2 patterns. In order to illustrate our method of examining electrophoretic patterns, the main features distinguishing the patterns of S. from those of M. are indicated: 1) There is a definite separation of the albumin from the α_1-globulin peak in S. but only an incomplete separation of these components in M. 2) The shape of the α_2-globulin peak of S. is symmetrical. The α_2-globulin peak of M., however, is asymmetrical showing a small amount of a protein component with a mobility slightly exceeding that of the main α_2-globulin peak, but not separating well from the latter. 3) The peak of α_2-globulin for S. is much broader than that

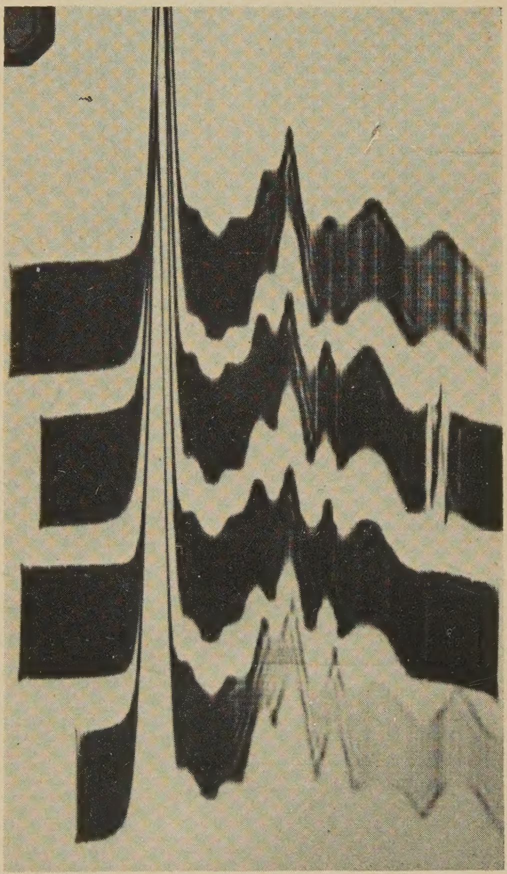


FIG. 2. Electrophoretic patterns of patient E. at different times. Dates from top to bottom: June 30, July 7 and 17 and Oct. 6, 1950. The peaks represent from left to right: albumin, α_1-globulin, α_2-globulin, α_3-globulin, β-globulin, fibrinogen, γ-globulin and remainder of δ-anomaly.

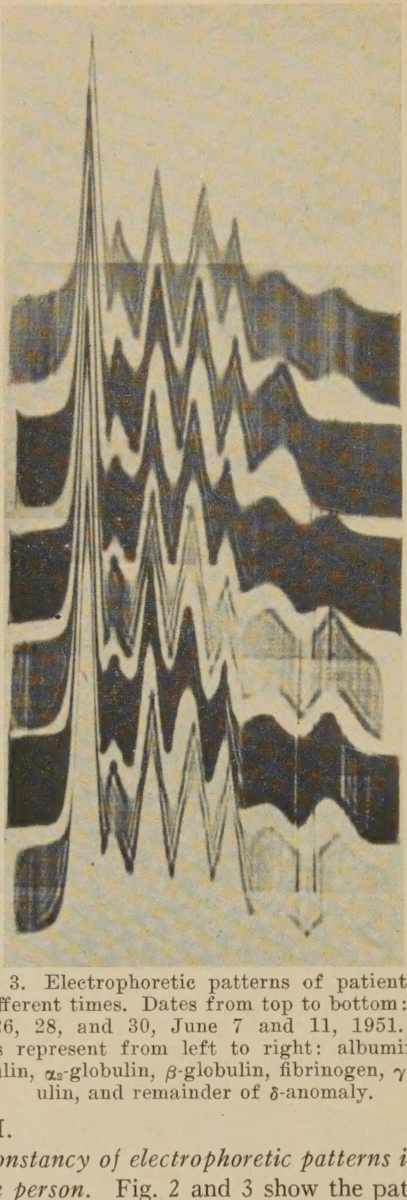


FIG. 3. Electrophoretic patterns of patient A.S. at different times. Dates from top to bottom: May 23, 26, 28, and 30, June 7 and 11, 1951. The peaks represent from left to right: albumin, α_1-globulin, α_2-globulin, β-globulin, fibrinogen, γ-globulin, and remainder of δ-anomaly.

of M.

Constancy of electrophoretic patterns in the same person. Fig. 2 and 3 show the patterns obtained in 2 patients. In each figure several patterns obtained from the same patient on different days are superimposed. In Table II the relative amounts of plasma proteins, as calculated from the patterns, are given. It is obvious that the general appearance of the electrophoretic patterns in the same individual remains remarkably constant over long periods of time, although the relative concentrations of the protein components may vary considerably during this time. Differences such as observed in Fig. 1 do not appear in the same person at different times. This *constancy* in the aspect of the electrophoretic patterns has been observed in a great number of individuals. Temporary deviations, however, have been noticed in a number of cases under the influence of various factors, such as abscess formation, ACTH treatment(6), and others. In most of these cases the patterns return to their original appearance after a lapse of time.

Individual electrophoretic patterns. Upon comparing the electrophoretic patterns from different individuals with each other it becomes obvious that it is hardly possible to find two persons whose patterns have the same characteristic features. This phenomenon is so pronounced that in many cases it is possible to identify individuals by their electrophoretic plasma patterns. Fig. 4 and 5 show 2 examples of patients with characteristic individual patterns.

Such individual patterns have been observed in a great number of other cases. In some individuals the plasma always contained an α_3-globulin which separated well from the α_2- and β-globulins, such as in patient E. (Fig. 2). On the other hand, plasma of some other persons never showed the presence of an α_3-globulin, as in patients B.M. and D.

TABLE II. Relative Amounts of Plasma Proteins in % of Total Protein.
Patients E. (top) and A.S. (bottom).

Date	Albumin	α_1-glob.	α_2-glob.	α_3-glob.	β-glob.	Fibr.	γ-glob.
6/30	49.4	3.4	5.0	9.0	14.8	5.8	12.6
7/ 7	49.1	4.5	4.9	9.5	12.8	6.6	12.6
17	47.9	5.7	5.6	9.2	12.8	7.0	11.8
10/ 6	44.0	5.7	5.5	10.1	15.7	8.3	10.7
5/23	31.4	10.0	19.0		18.4	11.6	9.6
26	30.7	7.9	21.0		17.6	10.2	12.6
28	34.5	9.2	19.3		19.0	9.0	9.0
30	38.1	8.4	19.8		17.9	7.9	7.9
6/ 7	36.8	8.5	20.9		18.0	9.3	6.5
11	34.3	9.7	18.8		17.4	12.6	7.2

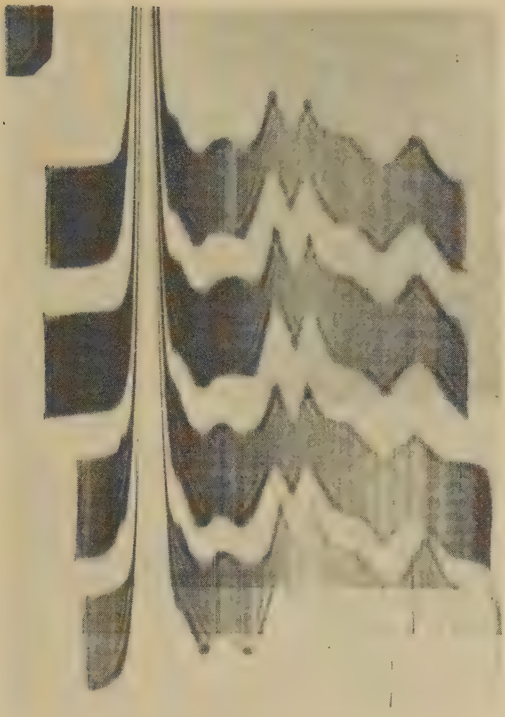


FIG. 4. Electrophoretic patterns of patient B.M. Dates from top to bottom: Dec. 7 and 9, 1950, Jan. 5 and 17, 1951.

(Fig. 4 and 5). In other individuals the α_3- and β-globulins did not separate from each other, yielding consistently one single, relatively high gradient at their place. Sometimes, the electrophoretic patterns of two persons were apparently very similar to each other, but a close examination of their structure always revealed characteristic differences, distinguishing the two individuals from each other, *e.g.* a patient (Y.) with seborrheic dermatitis produced electrophoretic patterns which were almost identical to those of patient D. (Fig. 5), with the difference, however, that all the patterns of Y. exhibited a much more pronounced α_1-globulin than those of D.

Our findings on the relationship between electrophoretic patterns and certain diseases will be reported later. It should be emphasized, however, that there is no evidence in our material for the existence of electrophoretic patterns characteristic for any of the diseases mentioned in this paper. Indeed, three patients with rheumatoid arthritis (Fig. 1, M.; 2, E.; and 5, D.) have entirely different electrophoretic patterns.

Patients as well as normal individuals show this diversity of the electrophoretic plasma patterns. The most characteristic features distinguishing normal individual patterns from each other are in general found in the α_1-, α_2- and α_3-globulins.

Summary. 1. The detailed examination of the contours and fine architecture of the electrophoretic patterns of human plasma in different individuals may reveal considerable dissimilarities between persons, even in those cases where the relative amounts of the plasma protein components, calculated from the electrophoretic patterns, are similar. The careful examination of the electrophoretic patterns, of the shape, symmetry or asymmetry, and width of each protein peak and of the tendency of adjacent protein peaks to separate from or to approach each other is considered to be of great importance to characterize a given pattern. 2. The general features and the appearance of the electrophoretic patterns in one and

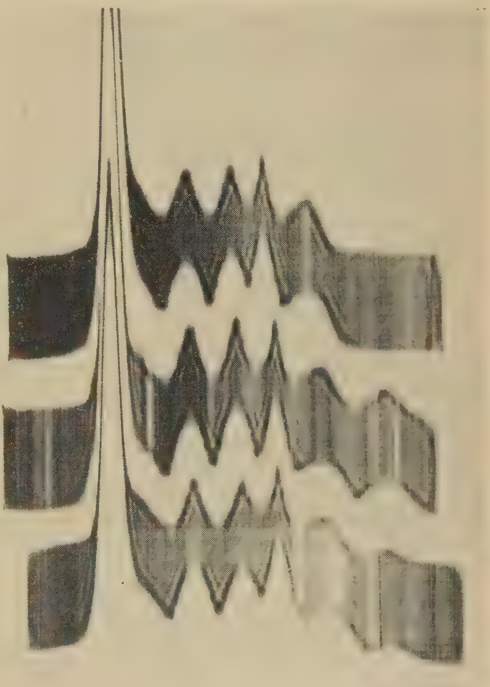


FIG. 5. Electrophoretic patterns of patient D. Dates from top to bottom: April 14, Sept. 2, 1950, and June 1, 1951.

the same person usually remain constant over long periods of time, although the plasma protein composition may vary considerably during this period. 3. Characteristic individual electrophoretic patterns of the plasma proteins have been observed in numerous cases of both normal individuals and patients with various diseases. The distinguishing characteristics encountered in individual electrophoretic patterns are independent of sex and age; in healthy persons they are confined mainly to the α-globulins; with only a few exceptions they are not related to the disease state in patients.

1. Gutman, A. B., The Plasma Proteins in Disease, *Adv. Protein Chem.*, 1948, v4, 155.

2. Wuhrmann, F., and Wunderly, C., *Die Bluteiweisskorper des Menschen,* Basle, 1952.

3. Longsworth, L. G., and McInnes, D. A., *J. Am. Chem. Soc.*, 1940, v62, 705.

4. Wiedemann, E., *Helv. Chim. Acta,* 1947, v30, 168.

5. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, v69, 119.

6. Bernfeld, P., Bonner, C. D., and Homburger, F., *Proc. of the 2nd Clin. ACTH Conf.*, Blakiston Co., 1951, v1, 282.

Received April 28, 1953. P.S.E.B.M., 1953, v83.

Growth Promotion in Carnivorous Protozoa by Substituted Purines.* (20380)

DANIEL M. LILLY, FRANCIS J. STERBENZ, AND VINCENT TARANTOLA.
(Introduced by George W. Kidder.)

From the Department of Biology, St. John's University, Brooklyn, N. Y.

Tetrahymena pyriformis(1) has been extensively used as a favorable animal microorganism for nutritional and biochemical studies(2-4). When *Tetrahymena* has been used as food for carnivorous protozoa an interference with their normal growth and reproduction has been noted. By the addition of small concentrations of naturally occurring substances to the culture medium, however, (together with the food organism) it has been possible to obtain satisfactory growth of several species of carnivorous protozoa. In the case of the hypotrichous ciliate, *Stylonychia pustulata,* in bacteria-free cultures growth was proportional to the number of *Tetrahymena* supplied as food, but failed immediately if a supplement, extracted from yeast or other plant materials, was omitted from the medium. The supplement was never obtained from animal tissues and attempts to substitute known vitamins were unsuccessful(5-7). Chemical properties of the supplementary factor, such as solubility in aqueous, but not in organic solvents, thermostability and photosensitivity were established. By chromatographic methods concentrates were produced which were active in dilutions as low as 0.1 μg per ml(8,9). In the case of the suctorians, *Tokophrya infusionum* and *Podophrya collini*[†] the interference with growth was manifested differently. The adult suctorians captured *Tetrahymena* readily with their tentacles and sucked out the cytoplasm of the ciliate. There was apparently no immediate need for a supplement in the medium as long as the number of food ciliates was limited. Thus Rudzinska was able to maintain *Tokophrya* for several years by carefully controlling the supply of *Tetrahymena*(10). It has long been known, however, that after abundant feeding on ciliates a large percentage of suctorians changed into giant forms which failed to reproduce normally(11). Under such conditions there was invariably a decline in the growth rate with eventual loss of the cultures. In bacteria-free cultures of suctorians feeding on *Tetrahymena* small concentrations of yeast

---

* Aided by a grant from the National Science Foundation.

[†] We wish to thank Dr. Harold E. Finley of Howard University for sending us the original culture of this organism.

extract were found beneficial in reducing the percentage of giantism and in maintaining cultures(12,13). In cultures of *Tokophrya* yeast nucleic acid was equally effective, but highly active concentrates of the supplementary factor required by *Stylonychia* were without effect on this suctorian. Conversely, it had previously been determined that yeast nucleic acid did not contain the *Stylonychia* factor. After a chemically defined medium for *Tetrahymena* had been developed(3), the mixture of purines and pyrimidines used in that medium was tested on *Tokophrya* with even better results than those obtained with nucleic acid. A combination of guanylic acid and uracil was also effective, but neither alone had a beneficial effect(14). These results were interpreted as indicating that the purine and pyrimidine requirement of *Tokophrya* was qualitatively similar to that of *Tetrahymena* but that these compounds were not available to the suctorian in proper quantities or proportions from the cytoplasm of the ciliate(15). It had been observed in this laboratory and elsewhere‡ that the nucleus of *Tetrahymena* was not taken in by *Tokophrya*.

The present report resulted from a study of the biological activity of purines in the carnivorous protozoa by methods similar to those used in *Tetrahymena*(16). In the first experiments on *Tokophrya* the triazolo purine analog, 8-azaguanine, acted as it did in *Tetrahymena*, as a metabolic inhibitor. When, however, tests were extended to the other suctorian, *Podophrya*, a growth promoting effect was noted. Recent trials on *Stylonychia* indicate that 8-azaguanine is also growth promoting for this ciliate and replaces the naturally occurring supplement previously found necessary.

Materials and methods. *Tetrahymena pyriformis* W was supplied to the carnivorous protozoa by two methods. Originally the food ciliates were cultured separately on slants containing 2% proteose peptone, 0.5% dextrose and 1.4% agar. After 4 days' growth the ciliates were suspended in sterile distilled water for addition to the experimental cultures. This method was continued for the studies on the *Stylonychia* supplementary factor and was used for the preliminary studies on the suctorians. Recently a chemically defined medium has been developed based on Medium A for *Tetrahymena*(3). The amino acids, purines and pyrimidines were diluted 10 times, dextrose and Tween were omitted, and a chelating agent (ethylenediamine tetraacetic acid) was added. The carnivorous protozoa were washed free of bacteria by a technic previously published(17). Cultures were maintained in tubes containing 5 ml of medium and were incubated in the dark at 26°C. When *Tetrahymena* were taken from slants relatively heavy suspensions were used as described elsewhere(6). In the chemically defined medium the food ciliates were introduced with the carnivorous protozoa, in a ratio of approximately 50 to 1, from a stock cul-

TABLE I. Effect on Giantism in Suctorians of Increasing Concentrations of Purines and Pyrimidines.

Medium	*Tokophrya infusionum,* %	*Podophrya collini,* %
Basal medium*	83	8.5
” ” and 3x†	33.8	32
” ” ” 6x	80	50
” ” ” 9x	100	56

* Contains 3 γ/ml guanylic acid and 1 γ/ml uracil.

† x = guanylic acid, 30 γ/ml; adenylic acid, 20 γ/ml; cytidylic acid, 25 γ/ml; uracil, 10 u/γ/ml.

TABLE II. Effect on Giantism in Suctorians of 8-Azaguanine, Guanylic Acid and Uracil.

Medium	*Tokophrya infusionum,* %	*Podophrya collini,* %
Basal medium*	87.3	9
” ” and uracil†	30	100
Basal medium and guanylic acid‡	1.3	
Basal medium, uracil and guanylic acid	20.9	100
Basal medium, uracil and 8-azaguanine§	100	
Basal medium, guanylic acid and 8-azaguanine	100	0
Basal medium, uracil, guanylic acid and 8-azaguanine	100	25
Basal medium and 8-azaguanine	100	0

* Contains 3 γ/ml guanylic acid and 1 γ/ml uracil. † 10 γ/ml. ‡ 10 γ/ml. § 0.18 γ/ml.

---

‡ Personal communication from Dr. Maria A. Rudzinska.

TABLE III. Effect of Substituted Purines on Growth of *Stylonychia pustulata* Feeding on *Tetrahymena*.*

μg/ml guanylic acid	Substituted purine	μg/ml substituted purine						
		100	10	1.0	0.1	.01	.001	0
0	8-Azaguanine		+	+	+	+	+	0
50	”		+	+	0	0	0	0
0	Theobromine	+	+	+	+	+	+	0
5	”	++	++	++	++	++	+	0
0	Theophylline	+	+	+	++	++	++	0
5	”	+	+	+	+	++	+	0
0	Caffeine	0	+	+	++	+	+	0
		400	200	100	50	25		0
1	Caffeine	0	+	+	+	+		0

* No growth without naturally occurring supplement or substituted purine.
0 = No growth; + = Significant growth; ++ = Approximately half maximum growth.

ture. Growth of *Tetrahymena* kept pace with the growth of the carnivorous organisms during the first 3 days, except when substances inhibitory for the food ciliate were added. The tubes were examined for recording results usually on the seventh day. The suctorians were counted in a zone just below the surface of the medium on the sides of the tube. Growth of suctorians was recorded in terms of numbers of organisms per low power field, 1.13 sq mm area. At the same time the percentage of giantism was determined. Giants were characterized by a size at least twice that of the normal form and by the absence of visible tentacles. Methods for recording growth of *Stylonychia* have been given previously(6) but in the work presented here only a rapid, routine grading of significant growth by plus marks was attempted.

Results. The average number of adult suctorians per low power field was approximately doubled by the same factors which decreased giantism. The effect on the percentage of giantism, however, was much more striking and was taken as a better indication of the influence on growth in these organisms. In *Tokophrya* addition of 30 γ per ml of guanylic acid and 10 γ per ml of uracil to the basal medium reduced giantism from 82.6% to 34.8%. The influence of increasing concentrations of purines and pyrimidines was then tested on both species of suctorians with the results given in Table I. In *Tokophrya* there was a favorable effect on growth with up to 3 times the concentration of nucleic acid components usually used for *Tetrahymena* but higher concentrations were inhibitory. Concentration is not the only factor influencing giantism, however, in this species as is shown by the high incidence of giantism in the most favorable concentration tested. Table II affords better evidence that the proportion of purine to pyrimidine is more important, since the least giantism occurred in *Tokophrya* when the ratio of guanylic acid to uracil in the medium was 13 to 1. The basal medium contains some guanylic acid and uracil to permit the growth of *Tetrahymena*. The guanine analog, 8-azaguanine, effectively reverses this ratio and increases giantism to the maximum.

In contrast with the findings in *Tokophrya*, the results with the other suctorian, *Podophrya* indicate that any addition of nucleic acid components increases the percentage of giantism. Even uracil has this effect. The guanine analog, however, completely reverses the condition with total elimination of giant forms. Although not shown in the tables, giantism in this species is also prevented by other guanine antagonists, caffeine, theobromine and theophylline. Concentrations from 0.1 to 100 γ per ml of these methylpurines are growth promoting for this suctorian.

In the hypotrich *Stylonychia* there is also a growth promoting effect with the same purine antagonists which benefit *Podophrya*. It should be noted, however, that the growth thus far obtained is not equal to that observed in concentrates from natural sources. Other factors are undoubtedly involved, but the guanine antagonism, even in these preliminary experiments, is quite clearly shown to be operative. These are the first known chemical compounds which have given positive results as substitutes for the naturally occurring supplementary factor. The indication that the growth promoting action of theobromine is slightly improved by the addition of guanylic acid suggests that a delicate purine balance is involved.

Discussion. The remarkable difference in the effects of 8-azaguanine in the two species of suctorians suggests that equally great variations in purine metabolism remain to be discovered in other organisms. *Tetrahymena pyriformis* has been unique among the ciliates in the facility with which it was first cultured in peptone solutions(2) and in its progressive yield of biochemical information on its metabolism(3). Yet the most striking peculiarity of *Tetrahvmena* when compared to other animals is its guanine metabolism(16). The indication that guanine metabolism is critically involved as a condition for growth in other protozoa may explain many of the difficulties which have hindered nutritional studies with animal microorganisms other than *Tetrahymena.* It now appears that *Tokophrya infusionum* has an optimum ratio of guanine to uracil considerably higher than that afforded by its method of feeding on *Tetrahymena.* Adding guanylic acid to the medium, therefore, improves growth while adding 8-azaguanine aggravates the interference. In *Podophrya collini,* on the other hand, the optimum purine intake is apparently exceeded by feeding on *Tetrahymena.* Hence quanylic acid aggravates the interference with growth while the guanine antagonist improves growth. The fact that uracil also increases the percentage of abnormal forms, of course, requires further study. Quite different from the suctorians by any morphological or physiological comparison, the hypotrich *Stylonychia pustulata* nevertheless responds to 8-azaguanine and the methylpurines as does *Podophrya.* Hence the phenomenon transcends the suctorian group of protozoa and may apply to many ciliates. The results with *Stylonychia* suggest that in this organism there is such a critical purine balance that the ingestion of a few *Tetrahymena,* with their peculiar guanine metabolism, is sufficient to prevent growth. The guanine antagonists apparently affect the balance to the extent that growth on the *Tetrahymena* diet is possible, although the optimum has not yet been achieved. In *Stylonychia* also there is a difference between the effect of 8-azaguanine and that of the methylpurines. It has been pointed out(16) that the methylpurines inhibit more than one enzyme system. Purine is the most easily affected but there is a possible effect on pyrimidine and other non-purine systems. A close similarity is noted between theobromine and the most active preparations of the *Stylonychia* supplementary factor previously concentrated from natural sources(15).

Summary. The guanine analog, 8-azaguanine, had a growth inhibiting effect on one species of suctorian, *Tokophrya infusionum,* and a growth promoting effect on another species, *Podophrya collini.* The compound induced abnormal giant forms in the former and prevented their appearance in the latter. Guanylic acid antagonized both effects. In the hypotrichous ciliate, *Stylonychia pustulata* 8-azaguanine promoted growth and substituted for a supplementary factor previously obtained from natural sources. The methylpurines, caffeine, theobromine and theophylline were also growth promoting in *Podophrya* and *Stylonychia.* The results may be explained on the basis of a purine balance in these protozoa. Apparently guanylic acid intake in other than optimum proportions can interfere with growth in the organisms studied.

---

1. Corliss, J. O., *Trans. Am. Micros. Soc.,* 1952, v71, 159.
2. Lwoff, A., *Ann. Rev. Microbiol.,* 1947, v1, 101.
3. Kidder, G. W., and Dewey, V. C., in *Biochemistry of Protozoa,* A. Lwoff, ed., Academic Press, 1951.
4. Kidder, G. W., *Ann. Rev. Microbiol.,* 1951, v5, 139.
5. Lilly, D. M., *Anat. Rec. Suppl.,* 1940, v78, 40.
6. ———, *Physiol. Zool.,* 1942, v15, 146.
7. Lilly, D. M., Pignataro, A., and Heilshorn, K., *Anat. Rec.,* 1949, v105, 512.
8. Lilly, D. M., Bulda, F. E., and Daly, J. J., *Anat. Rec.,* 1951, v111, 112.
9. Lilly, D. M., Daly, J. J., and Tarantola, V., *Proc. Soc. Protozool.,* 1952, v3, 13.
10. Rudzinska, M. A., *Science,* 1951, v113, 10.

11. Collin, B., *Arch. Zool. Exp. et Gen.*, 1912, v51, 1.

12. Guilcher, Y., Thesis, University of Paris, France, 1950.

13. Lilly, D. M., Fleming, P. J., Pedagno, C. F., and Gilmore, J. P., *Anat. Rec.*, 1951, v111, 62.

14. Lilly, D. M., and Gilmore, J. P., *Anat. Rec*, 1952, v113, 64.

15. Lilly, D. M., *Ann. N. Y. Acad. Sci.*, 1953, in press.

16. Kidder, G. W., and Dewey, V. C., *J. Biol. Chem.*, 1949, v179, 181.

17. Kidder, G. W., Lilly, D. M., and Claff, C. L., *Biol. Bull.*, 1940, v78, 9.

Received May 1, 1953. P.S.E.B.M., 1953, v83.

III. Chemotherapy of Tumors in Rats with Certain Ethylenephosphoramides.*† (20381)

M. L. CROSSLEY, J. B. ALLISON, R. P. PARKER, E. KUH, AND D. R. SEEGER.

From Bureau of Biological Research, Rutgers University, New Brunswick, N. J., and The Calco Chemical Division, American Cyanamid Company, Bound Brook, N. J.

As a continuation of our investigation of the therapeutic effectiveness of ethylenimines in the treatment of transplantable tumors in rats(1,2), we report here results obtained with N,N′,N″ - Triethylenephosphoramide, (I), N,N-Diethyl-N′,N″-diethylenephosphoramide, (II), N-Pentamethylene-N′,N″-diethylenephosphoramide, (III), N-(3-Oxapentamethylene) - N′,N″ - diethylenephosphoramide, (IV), N-Tetramethylene-N′,N″-diethylenephosphoramide, (V), N,N′,N″-*tris* (Tetramethylene)phosphoramide, (VI), N,N′,N″-Triethylenethiophosphoramide, (VII) and N - (3 - Oxapentamethylene) - N′,N″ - diethylenethiophosphoramide, (VIII).

Materials and methods. The compounds were synthesized in the Pharmaceutical Research Laboratory of The Calco Chemical Division of The American Cyanamid Co. Their structural formulas are shown in Fig. 1. They are colorless, crystalline compounds. (I) melts at 41°C, is extr. soluble in water, very soluble in alcohol, ether and acetone. (III) boils at 95°C/.15 mm Hg and is very soluble in water, benzene, toluene and ether. Both (I) and (III) are very hygroscopic. (IV) melts at 62.5-64°C, is very soluble in water and is soluble in benzene, toluene and ether. (VII) melts at 51.5°C and its solubility in water at room temperature is 19 g in 100 ml. It is soluble in benzene and ether. (VIII) melts at 75-77°C, is slightly soluble in water and very soluble in benzene, toluene and hot petroleum ether. Isotonic saline solutions of the compounds were prepared to contain the desired dose in .2 ml. At first solutions were prepared daily, but equally good results could be obtained with solutions prepared once a week, provided that solutions were kept at about 5°C. One dose was given daily by intraperitoneal injection. In certain cases the subcutaneous route was used for comparison and similar results were obtained. Treatment was given daily throughout the experiment which varied from 2 to 9 weeks. Young male rats, averaging about 150 g were used. In each experiment there were, generally, 4 groups of 6 to 8 rats each; normal controls (N.C.), drug controls (D.C.), tumor controls (T.C.), and tumor treated (T.T.). They were group pair-fed throughout period of treatment. In certain cases the W.B.C. and differential counts were made on all animals in the 4 groups, prior to implantation of the tumor, just before beginning the course of treatment and when the drug was discontinued or the experiment terminated; in certain experiments they were made, also, sometime

---

* Presented at International Congress of Pure and Applied Chemistry, New York City, 1951; at Annual Meeting of Am. Assn. for Cancer Research, New York City, 1952; and, at Meeting of Am. Chem. Soc., Atlantic City, 1952.

† This investigation was supported by grants from The Damon Runyon Memorial Fund and The Calco Chemical Division of The American Cyanamid Co.

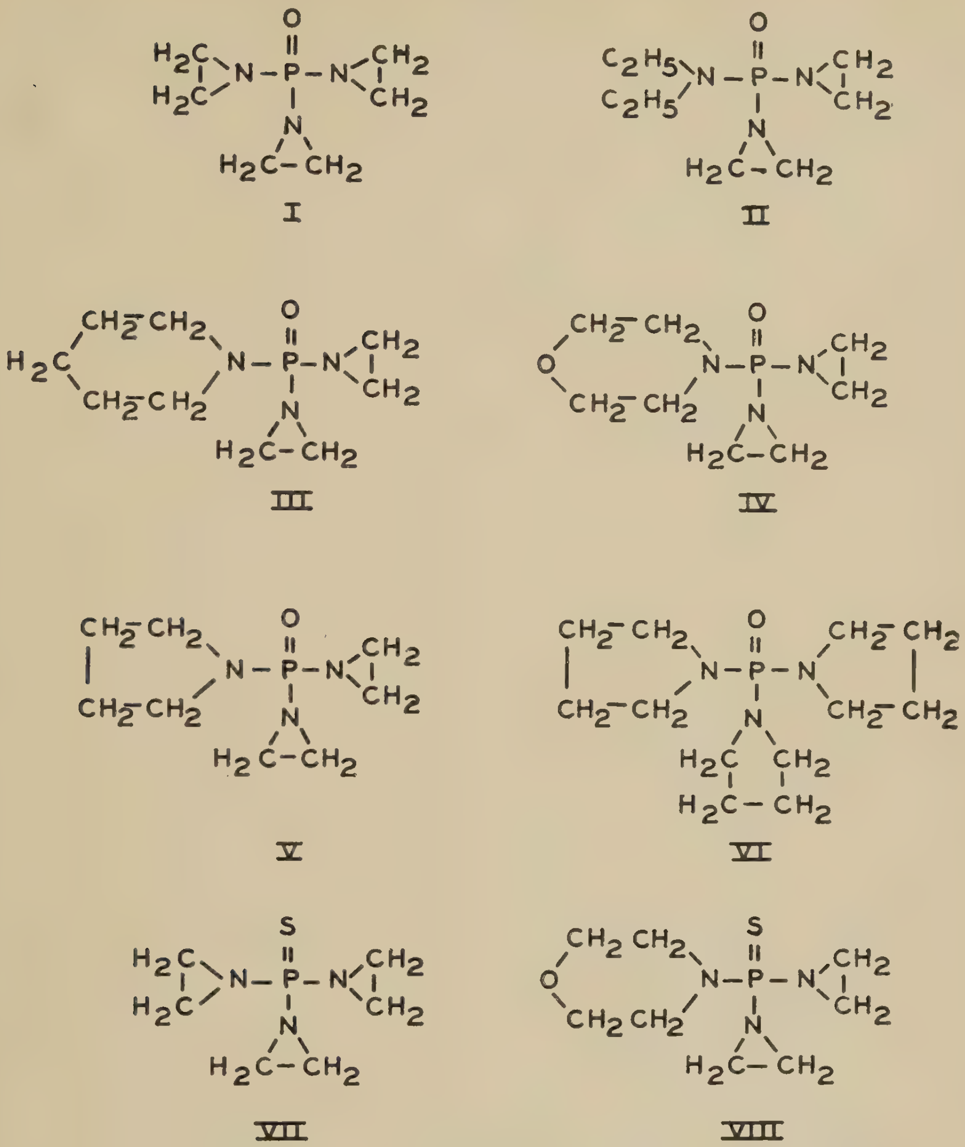


FIG. 1. Structural formulas of products mentioned in the text by numbers.

after the drug treatment was discontinued. The rats were fed the 18% casein diet described by Wase and Allison(3). At the end of treatment the animals in which the tumor had regressed completely were given fox chow, *ad lib.* and examined periodically to find out if they were cured and if they were resistant to growth of further transplants of the tumor. Sarcoma 231 in King A rats, Flexner-Jobling carcinoma and Walker carcinoma 256 in Sprague-Dawley rats and Sarcomas 3 and 7 in Lewis rats were used. We are indebted to Margaret Reed Lewis for the latter 2 tumors which were originally induced by methylcholanthrene in the Lewis rat. The methods for implantation of tumors, measuring growth and determining resistance of cured rats were essentially the same as previously described (1). Groups of rats, treated and controls, were killed at definite periods after transplantation of tumors and certain organs weighed and studied histologically and histochemically.

The weight of organs was expressed in g/100 g of gross body weight.

Results. The initial dose of N,N′,N″-Triethylenephosphoramide (I), was the maximum amount which, when injected intraperitoneally, once daily, in normal Sprague-Dawley rats for 7 days, produced no significant leukopenia. Optimum dose of 2,4,6-Triethylenimino-s-triazine for the rat was about .04 mg/kg/D. For (I) it was .43 mg/kg/D. With this dose the W.B.C. of treated rats did not differ materially from that of the control group and there were no manifestations of toxic symptoms. Average increase in body weight was about the same in treated and untreated pair-fed groups.

Preliminary results had shown that ethylenephosphoramides inhibited growth of certain tumors in mice and rats(4-8). Also, they were effective in transplantable mouse leukemia(9).

In 5 experiments with Sarcoma 231 in young King A rats, 150-175 g body weight when treatment began, the tumor regressed completely in 2 of 13 controls and in 26 out of 37 treated. The dose ranged from .4 to .6 mg/kg/D, injected once daily for 2 to 6 weeks. All rats in which the tumor regressed completely were permanently cured. They were challenged several times within a period of 8 to 10 months and remained resistant to growth of grafts. The animals appeared to be similar in well-being to normal controls of their respective groups of same age. Treatment was started when the tumor was well established, usually about 7 days after implantation tumor size averaged 250 to 300 mm^2. In certain experiments treatment was delayed until size of tumor was 800 to 1200 mm^2. In such cases treatment did not begin until about 12 days after implantation. There were no marked differences in number of animals cured or in time required for tumors to regress completely. In all groups treated the tumor continued to grow for a few days at about the same rate as controls, then the majority stopped growing and abruptly regressed with regression continuing to completion. In most cases where the tumors did not regress there was only a slight inhibition in rate of growth followed by acceleration in spite of treatment. Occasionally a tumor which had regressed almost to completion, reversed its course while under treatment and became refractory to the drug.

Compounds (I) and (II) were compared at dose level of .4 mg/kg/D in a few King A rats bearing Sarcoma 231 and preliminary results indicated that this dose, (I) was probably a more effective therapeutic agent than (II). Ten young male King A rats bearing Sarcoma 231 were treated with (III), 5 with .8 mg/kg/D and 5 with 1.2 mg/kg/D. Treatment began 8 days after implantation and continued 18 days. There were 3 regressions in rats getting the smaller dose and 2 with the higher. The 2 treated groups were pair-fed with a group of 5 tumor controls. There were no regressions in the controls and all of the animals died of tumor within 21 days. The King A rat was no longer available and further work with Sarcoma 231 had to be abandoned.

Fifty-eight male Sprague-Dawley rats bearing Flexner-Jobling carcinoma were treated with doses of (I) varying from .30 to 1.26 mg/kg/D. The tumor regressed completely in 42. In 26 tumor controls there was 1 spontaneous regression. There were no significant differences in the number of regressions at the several dose levels. The product was effective over a much wider therapeutic range than T.E.M. Animals that were cured appeared normal except that their testes were smaller than those of controls. Effect on testes varied with dose and length of the period of administration of the product. Results obtained with normal animals receiving same doses were essentially the same as with treated tumor-bearing rats. Weights of testes and other organs in g/100 g of gross body weight are shown in Table I.

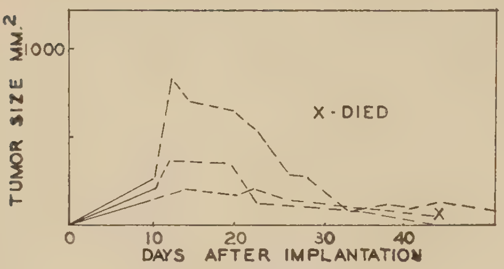
Unlike the King A rat, negative takes were not always obtained in Sprague-Dawley rats on first challenge after complete regression of Flexner-Jobling carcinoma. In most cases tumors grew to relatively small size and then regressed spontaneously. In a few instances the tumor grew to a medium size and became static. It was then refractory to treatment. In certain of these cases the tumor was removed surgically and found to be viable. The

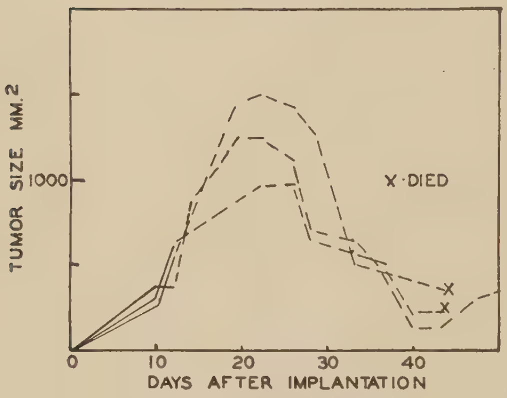
TABLE I. Effect of Tumor and Drug on Certain Organs of the Rat.

Rat	Group	Drug	Dose, mg/kg/D	Days treated	% BW change	Wt of organs in g/100 BW						
						Liver	Thymus	Adrenals	Kidneys	Spleen	Testes	S.V.
						S.D.–F.J.						
NC	(6)*			44		4.0	.17	.01	.73	.21	1.04	.26
TC	(4)			24		5.6	.18	.03	1.07	1.25	1.51	—
DC	(6)	I	.47	45		3.6	.17	.02	.79	.50	.77	.23
TT	(8)	I	.44	45		3.6	.15	.02	.67	.27	.59	.18
"	(6)	I	.85	46		3.7	.14	.02	.67	.41	.36	.28
NC	(11)			45	+ 77	3.5	.16	.02	.65	.25	.94	.17
TC	(6)			40		3.7	.16	.02	.65	.44	1.1	.18
TT	(2)	III	.18	52	+ 58	3.4	.13	.01	.80	.29	.87	.17
"	(3)	III	.37	52	+ 57	3.3	.14	.02	.67	.28	.78	.18
"	(2)	III	.93	52	+ 50	3.9	.10	.02	.72	.26	.62	.15
"	(12)	III	.90	43		3.2	.15	.02	.68	.35	.74	.20
"	(12)	III	.90	43		3.3	.14	.02	.67	.35	.71	.21
"	(3)	III	1.40	52	+ 51	3.5	.11	.02	.78	.28	.48	.22
NC	(6)			45	+ 97	4.4	.16	.02	.75	.28	1.06	.17
TC	(3)			24	+ 29	5.3	.14	.03	.91	1.65	1.20	.09
TT	(4)	IV	.21	51	+ 95	4.0	.12	.02	.86	.29	.90	.18
"	(5)	IV	.43	51	+ 94	4.4	.13	.02	.78	.33	.86	.16
"	(3)	IV	1.00	51	+ 80	4.0	.14	.02	.75	.25	.52	.18
"	(3)	IV	1.67	51	+ 80	3.8	.09	.02	.83	.35	.38	.16
NC	(6)			40		4.6	.21	.02	.80	.41	1.09	.23
TC	(6)			40		4.9	.21	.02	.85	.80	1.11	.19
DC	(4)	V	.75	55		3.0	.17	.02	.67	.19	.57	.23
"	(4)	V	1.01	42		4.8	.17	.02	.66	.46	.74	.18
TT	(4)	V	.94	42		4.5	.18	.02	.80	.53	.70	.16
"	(4)	V	1.09	56		3.4	.15	.02	.59	.38	.44	.21
DC	(4)	VI	1.12	36		4.0	.25	.01	.78	.38	1.09	.24
TT	(4)	VI	.90	36		4.9	.15	.02	.84	1.12	1.07	.12
NC	(4)			53	+ 98	3.7	.13	.01	.73	.36	.92	.21
TC	(4)			51	+ 53	3.8	.11	.02	.71	.59	1.01	.18
DC	(3)	VII	.43	53	+104	3.6	.15	.02	.79	.25	.61	.21
TT	(4)	VII	.33	51	+ 52	3.6	.09	.02	.71	.45	.58	.21
DC	(3)	VIII	.82	53	+109	3.7	.14	.01	.79	.30	.66	.21
TT	(3)	VIII	.64	51	+ 48	3.3	.08	.02	.70	.41	.64	.21
						S.D.–W. 256						
NC	(2)			14		3.2	.20	.02	.75	.27	1.18	.16
TC	(3)			14		6.7	.12	.03	.78	1.66	1.18	.08
TT	(8) not reg	VII	.49	29		6.6	.06	.04	.88	1.26	1.03	.11
"	(3) reg	VII	.47	29		4.0	.11	.02	.82	.42	1.11	.36
						L.–S–3						
NC	(4)			16		4.0	.20	.02	.90	.23	1.06	.24
TC	(4)			16		4.7	.18	.01	.84	.59	1.03	.07
DC	(2)	I	.58	9		4.0	.25	.03	.85	.20	1.26	.16
TT	(3)	I	.60	9		4.5	.12	.03	.97	.40	1.26	.05
						L.–S–7						
TC	(3)			17		4.7	.15	.02	.81	.47	1.02	.03
TT	(3)	I	.60	17		4.8	.09	.02	.85	.29	.90	.03

* No. of rats included in the avg results.

rats on recovery from the operation were still susceptible. In other cases the small static tumors were capsules filled with a viscous liquid or cheesy solid containing no viable tumor cells. Once resistance to growth of tumor was established it appeared to be permanent. Animals were challenged periodically up to 1 year after they first proved to be resistant and the results were negative. To ascertain if age of rat influenced development and response to treatment of the tumor 7 adult male rats, averaging about 300 g and bearing the carcinoma were given a dose of .39 mg/kg/D of (I) once daily for 15 days, treatment commencing 10 days after tumor implantation. Complete regressions occurred





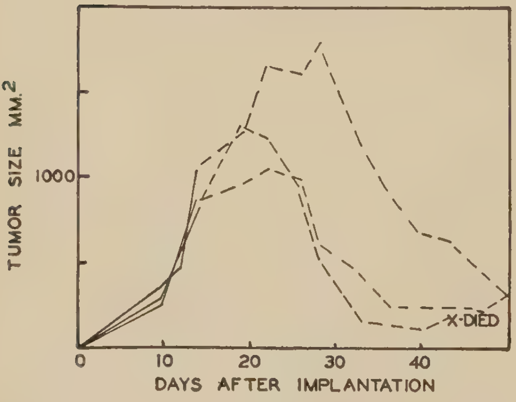


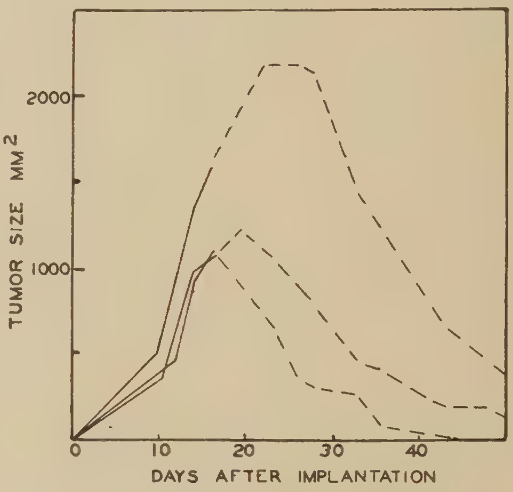
FIG. 2A (top). Growth curves of F-J carcinoma treated with .38 mg/kg/D (I), once daily from 10th day after implantation of the tumor. Period of treatment is indicated by broken lines.

FIG. 2B (center). Growth curves of F-J carcinoma treated with .42 mg/kg/D of (I), once daily from 12th day after implantation. Broken lines indicate period of treatment.

FIG. 2C (bottom). Growth curves of F-J carcinoma treated with (I), from the 14th day after implantation, dose .35 mg/kg/D. Broken lines indicate period of treatment.

in 4 during the treatment and in 2 of the remaining 3 during the 11 days immediately following the course of therapy. No regressions occurred in untreated tumor controls. Similar results were obtained in other experiments using adult rats and it appeared that the age of the rat did not play an important role in the development and regression of the tumor.

In order to determine the effects of treatment at different stages of tumor growth, 16 rats, that had been implanted at the same time



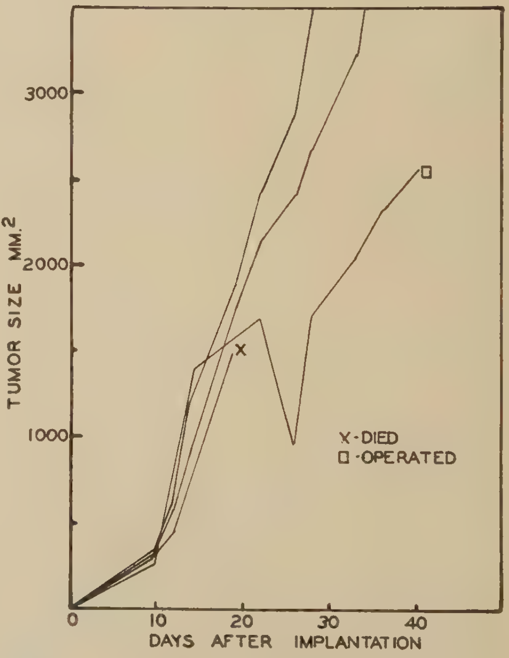


FIG. 2D (top). Growth curves of F-J carcinoma treated with (I) from 16th day after implantation, dose .30 mg/kg/D. Broken lines indicate period of treatment.

FIG. 2E (bottom). Growth curves of F-J carcinoma in group pair-fed untreated controls.

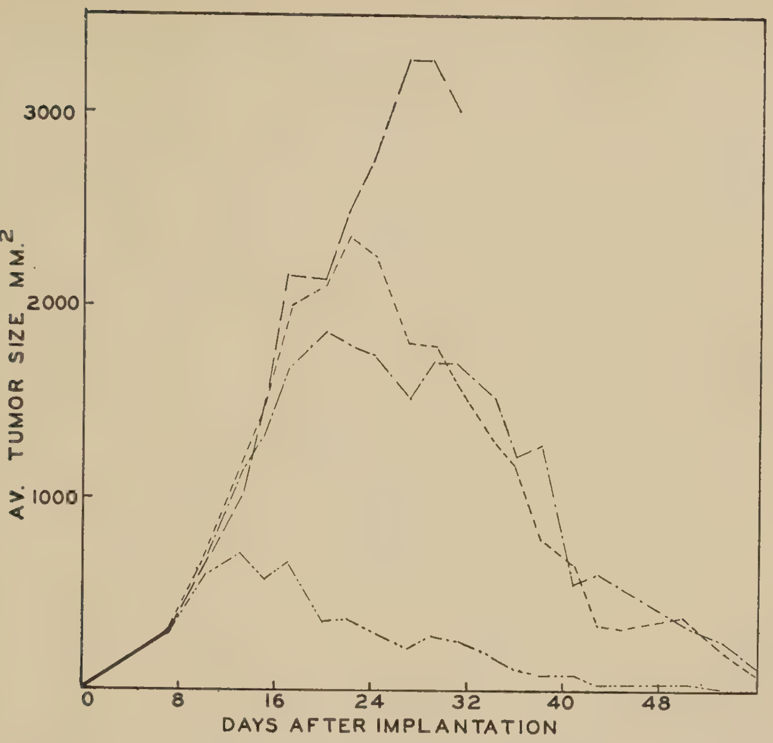
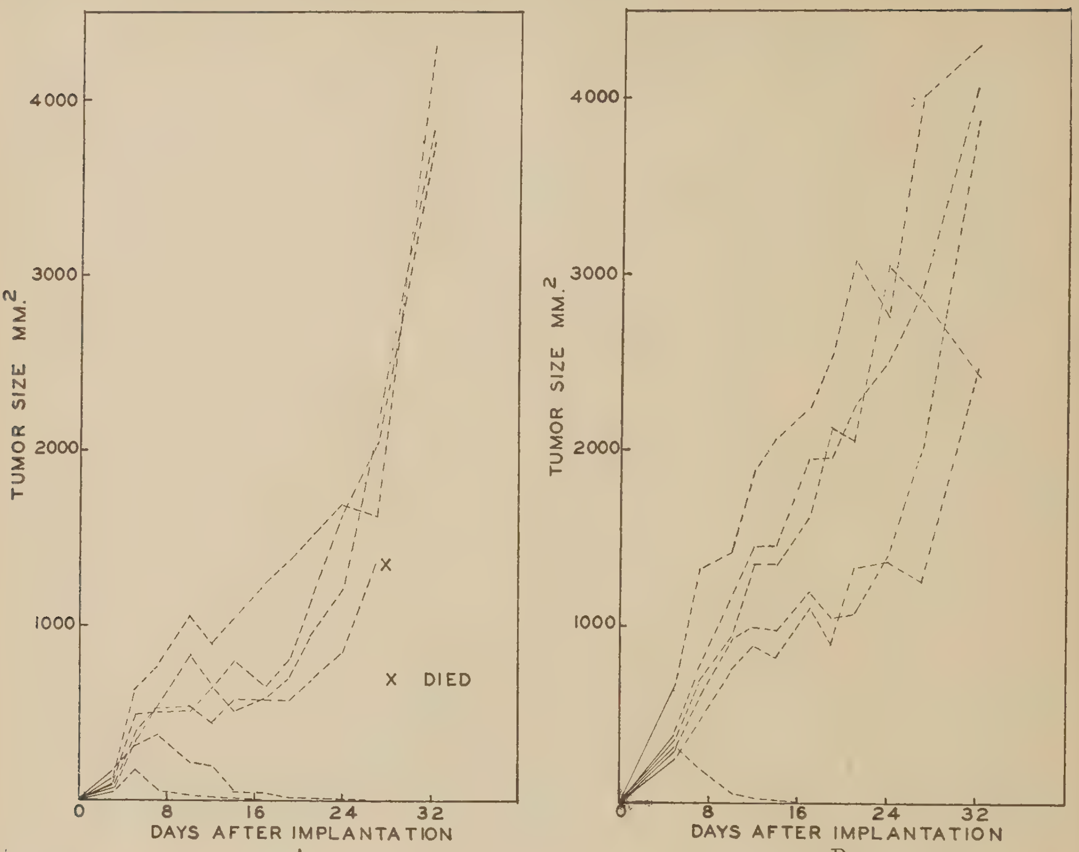


FIG. 3. Average growth curves of F-J carcinoma in pair-fed groups of S-D rats treated with different doses of (IV), once daily. (a) ——, untreated controls; (b) – –, .2 mg/kg/D; (c) —•—, .4 mg/kg/D; and (d) —••—, 1 mg/kg/D. The solid lines indicate period without treatment.

with the carcinoma and having well established tumors were chosen: 4 for controls and 12 for treatment with (I). The 12 animals were divided into 4 groups of 3 each. Therapy was started with the first group 10 days after implantation of tumor; the second, 12 days; the third, 14 days; and the fourth, 16 days. After the first few days of therapy, growth of tumors stopped in all groups and regression set in. This proceeded at a fairly rapid rate at first, particularly in the large tumors, then slowed as tumors became small. There was no significant difference in time required for complete regression of tumors in the rats of the different groups. Comparative behavior of the tumors in the 4 treated groups is illustrated in Fig. 2A-2E.

Fifty-four Sprague-Dawley rats bearing Flexner-Jobling carcinoma were treated with (III) in doses varying from .18 to 1.4 mg/kg/D given once daily for 38 to 53 days. Complete regressions occurred in 36 animals. There was no marked difference in therapeutic effectiveness of the different doses. Higher doses caused greater retardation of tumor growth during the first few days of treatment but the time required for complete regression of the tumor was about the same as with lower doses. Testes weight/100 g body weight decreased with increasing dose. In general, therapeutic range was wider than that of (I). Intraperitoneal and subcutaneous routes were compared at same dose level and results were about the same. With a dose of .74 mg/kg/D there were 5 regressions out of 7 in 53 days in each case. In another experiment, using 24 rats, 12 for each route of administration with a dose of .90 mg/kg/D there were, in 43 days of treatment, 6 regressions in group receiving drug subcutaneously and 8 in that getting it intraperitoneally. In 14 tumor controls there were 2 spontaneous regressions.

Product (IV) produced results similar to those obtained with (III). In 35 treated rats there were 29 complete regressions. It was used in doses varying from .2 to 1.7 mg/kg/D.



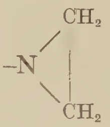
A

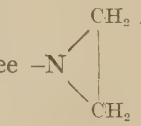
B

FIG. 4A. Growth curves of Walker 256 tumors in S-D rats, treated with .47 mg/kg/D of (VII). The broken lines indicate period of treatment.

FIG. 4B. Growth curves of Walker 256 tumor in S-D rats, treated with .50 mg/kg/D of (VII). Broken lines indicate period of treatment.

Within this range the dose did not seem to influence significantly total number of regressions. As in other cases mentioned the testes weight dropped with increasing dose. In general, this compound appeared to be the least toxic of the ethylenephosphoramides tested. Average growth rate curves for groups treated with different doses of (IV) are shown in Fig. 3. With the smallest dose the tumor grew during the first week at about the same rate as in untreated animals but after this, growth stopped and regression took place, continuing as in animals treated with higher doses.

Compound (V) in which one azetidyl group was substituted for one  $-\mathrm{N}\left\langle\begin{array}{l}\mathrm{CH_2}\\ |\\ \mathrm{CH_2}\end{array}\right.$ behaved like (III) and (IV). There were 8 regressions in 12 treated rats and none in 6 untreated controls. The dose was about 1.0 mg/kg/D.

In (VI), all three  $-\mathrm{N}\left\langle\begin{array}{l}\mathrm{CH_2}\\ |\\ \mathrm{CH_2}\end{array}\right.$ groups were replaced by azetidyl groups. The product was wholly inactive against tumor and did not influence weight of testes.

Products (VII) and (VIII) behaved, in general, like (I) and (IV). Treatment of Sprague-Dawley rats with Flexner-Jobling carcinoma with doses of .50 to .86 mg/kg/D of (VII) once daily gave 11 regressions in 17 treated rats while there were 2 in 14 untreated controls. There were 5 regressions in 8 treated with .94 mg/kg/D of (VIII).

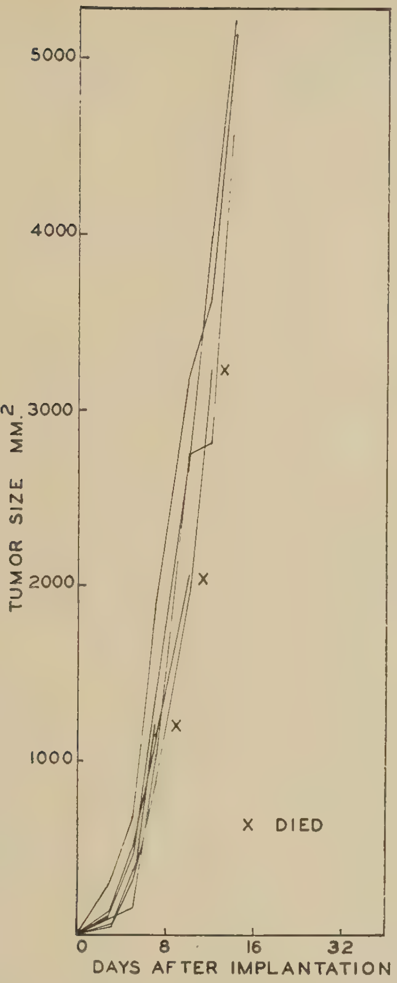


FIG. 4C. Growth curves of the Walker tumor in untreated S-D rats serving as pair-fed controls for 4A and 4B.

Preliminary results with the Walker 256 carcinoma in Sprague-Dawley rats indicated that animals bearing this tumor must be treated soon after implantation to obtain retardation and regression of tumor growth. In 12 rats treated with (VII), 6 were treated with .47 mg/kg/D for 29 days, treatment beginning 3 days after implantation of tumor. There were 2 regressions. The remaining 6 were treated with .50 mg/kg/D for 27 days, treatment beginning 5 days after tumor implantation. There was but 1 regression in this group. It will be seen from growth curves in Fig. 4A-4C that the drug was less effective in retarding growth in the second group than in the first and after the period of retardation growth of the majority of tumors increased in spite of continued treatment.

Fifty-four Lewis rats bearing Sarcomas 3 and 7 were treated with (I), (III) and (VIII) without success. These compounds, used in doses of .52 to 1.6 mg/kg/D, did not retard growth of these tumors. Treatment was started 3, 5 and 7 days after implantation. Tumor growth was about the same in all cases and did not differ significantly from that in untreated controls. The ethylenephosphoramides at the lower dose levels with which good therapeutic results were obtained in Sprague-Dawley rats affected testes only slightly and when the drug administration was discontinued the weight of testes became normal. With the largest doses the testes weight was only about 35 to 40% of normal and did not recover completely when treatment was discontinued. Effect of different doses on weight of testes in a given time is shown for compound (IV) and T.E.M. in Table II.

Action on testes occurs with all of the ethylenimines, in varying degrees. N,N′,N″-Triethylenimino-s-triazine, T.E.M., in relatively large doses, causes testicular damage (10). However, damage is slight with minimum therapeutic doses given to rats for several weeks. The influence of dose of T.E.M. on weight of testes of the Sprague-Dawley rat is shown in Table II. There appears to be a lag period in manifestations of the action of ethylenephosphoramides on both tumor and testes. The length of this period varies with dose of drug. With N,N′,N″-Triethylenephosphoramide (I, T.E.P.A.) at a dose level of .52 mg/kg/D, weight of testes remains normal for 14 to 16 days of therapy and then falls fairly rapidly as treatment continues. This is shown for 3 strains of rats in Fig. 5.

Results of histological and histochemical examination of several tissues as well as effect on pituitary activity will be reported later. Preliminary clinical results with the triethylenephosphoramides were published in two papers recently(11,12).

Growth of transplantable tumors we used was always accompanied by enlargement of spleen and by neutrophylic leukocytosis. Weight of spleen and the extent of neutrophy-

TABLE II. Effect of Dose on Weight of Testes.
(S.D.-F.J.)

Rat	Group	Drug	Dose, mg/kg/D	Days treated	Days off drug	% BW change	g*T/ 100 BW
NC	(4)				63	+138	.99
DC	(3)	IV	.24	64		+168	.84
”	(6)	”	.45	64		+166	.66
”	(6)	”	1.11	66		+142	.43
”	(2)	”	1.76	66		+118	.34
NC	(4)				149	+172	.95
DC	(2)	”	.24	64	85	+156	1.06
”	(1)	”	1.63	66	83	+157	.47
NC	(4)					+112	1.12
DC	(3)	T.E.M.	.04	42		+ 78	1.07
”	(5)	”	.10	42		+ 68	.64
”	(2)	”	.19	15	27	+ 63	.37

* g of testes.

lia corresponded to size of tumor. When tumors regressed completely, by treatment of rats or spontaneously, and rats were cured, both spleen and white blood-cells returned to normal. In those animals not cured of tumor, the spleen remained enlarged and neutrophylia persisted in spite of prolonged treatment. Tumors which did not respond to treatment never regressed spontaneously. Hypertrophy of the spleen and neutrophylia as accompaniment of tumor growth in mice and rats have been reported by others(13,14). Average weights of the spleens of tumor-bearing rats, treated and untreated, in comparison with pair-fed normal groups are given in Table I. The effect of growing and regressing tumors on white blood-cells is shown in Table III. The data express comparative conditions which existed at the time when experiments were terminated.

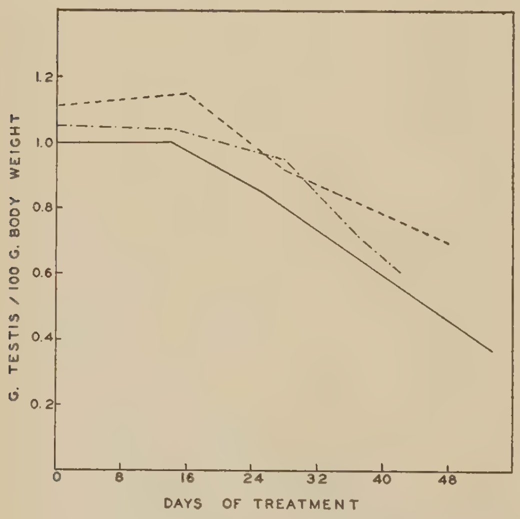
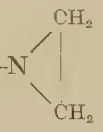


FIG. 5. Average weight curves of testes of S-D, Wistar and Lewis rats given .52 mg/kg/D of (I), and sacrificed in groups of 6 at definite periods of time for comparison with normal groups. The 0 values represent averages for normal groups. The —•—, represents S-D rats; ——, Lewis and the solid curve Wistar rats.

Discussion. Regression of tumors in treated rat did not seem to differ essentially from that which occurred occasionally without treatment and the immunity which resulted was essentially the same in effectiveness and permanence. It would seem probable that some metabolic imbalance helped to initiate a change in the growing tumor which caused it, not only to stop growing, but to regress. This was enhanced by ethylenimine drugs. Tumors which were not affected by the drugs, did not regress spontaneously. Host-tumor relationship must vary considerably. The results did not offer a convincing argument for oncolytic drug specificity.

Summary. 1. About 70% of the King A rats with Sarcoma 231 and 72% of the Sprague-Dawley rats with Flexner-Jobling carcinoma were cured by treatment with N,N′,N″-Triethylenephosphoramide (I). 2. The replacement of one

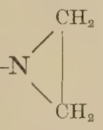
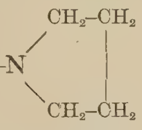


$-N\left\langle\begin{matrix}CH_2\\ |\\ CH_2\end{matrix}\right.$

group with a piperidyl, or morpholyl or azetidyl group did not affect the oncolytic properties significantly but did reduce the effect on testes; with a dose

TABLE III. Effects of Tumor Growth and Regression on White Blood-Cells of the Rat.

Rat–Tu.	Rat group	WBC	Polys.	Lymph
SD–FJ	NC (19)	18517	15	84
	TC (8)	47736	49	46
	TT reg. (34)	16012	19	79
	TT unreg. (11)	42133	50	47
KA–231	NC (6)	18550	24	72
	TC (6)	75000	68	28
	TT reg. (6)	12483	28	70
	TT unreg. (4)	63875	74	23
L–S-3	NC (6)	18567	19	80
	TC (6)	32800	40	56
	TT (9)	29833	52	47
L–S-7	TC (6)	33983	54	45
	TT (6)	27400	52	46

of .85 mg/kg/D of (I) given once daily for 45 days weight of testes of Sprague-Dawley rat was only about 35% of that of the normal controls, with a similar dose of (III) given in the same way for the same time it was about 79% of normal. Similarly, with a dose of .44 mg/kg/D of (I), once daily for 45 days the weight of the testes was 57% of normal while with a similar dose of (IV) given for 51 days weight of testes was 81% of normal. 3. The Flexner-Jobling tumor in the Sprague-Dawley rat regressed completely in about 70% of the animals treated with (III) and in about 80% of those treated with (IV). 4. At doses of about .2 mg/kg/D of (III) and (IV) good therapeutic results were obtained and weight of testes was but slightly below normal; when drug treatment was discontinued testes weight became normal. 5. The substitution of all three  $-N\begin{matrix}CH_2\\ \vert\\ CH_2\end{matrix}$ groups in (I) by  $-N\begin{matrix}CH_2\text{–}CH_2\\ \vert\\ CH_2\text{–}CH_2\end{matrix}$ groups resulted in compound (VI) which was inactive. 6. Compound (V) behaved like (III) and (VII), and (VIII) like (I) and (IV). 7. T.E.M. at a dose level of .04 mg/kg/D, cured 75% of the rats treated and weight of testes was normal. 8. Spleen enlargement and neutrophylic leukocytosis accompanied growth of all tumors studied. Animals recovered from both conditions when tumors regressed completely. 9. Sarcomas 3 and 7 in the Lewis rat did not respond to treatment with any of the ethylenephosphoramides nor with T.E.M. 10. The Walker tumor in the Sprague-Dawley rat responded to treatment better when therapy was started early; 3 days after implantation instead of later.

We acknowledge with deep gratitude our indebtedness to Rhoda Slovik, Dolores Turowski, Miriam Brush and Ruth Partridge for technical assistance with the biological experiments.

---

1. Crossley, M. L., Allison, J. B., Wainio, W. W., and Muenzen, J. B., *J. Nat. Cancer Inst.*, 1951, v12, 305.

2. Crossley, M. L., Allison, J. B., and Muenzen, J. B., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 452.

3. Wase, A. W., and Allison, J. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 147.

4. Buckley, S. M., Stock, C. C., Parker, R. P., Crossley, M. L., Kuh, E., and Seeger, D. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 299.

5. Stock, C. C., Buckley, S. M., Clarke, D. A., Parker, R. P., Crossley, M. L., Kuh, E., and Seeger, D. R., *Cancer Res.*, 1952, v12, 300 (Abstr.).

6. Sugiura, K., and Stock, C. C., *Cancer Res.*, 1952, v12, 300 (Abstr.).

7. Crossley, M. L., Allison, J. B., Parker, R. P., Kuh, E., and Seeger, D. R., *Cancer Res.*, 1952, v12, 256 (Abstr.).

8. Personeus, G., Halliday, S. L., McKenzie, D., and Williams, J. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 614.

9. Burchenal, J. H., Johnston, S. F., Stock, C. C., Parker R. P., Crossley, M. L., Kuh, E., and Seeger, D. R., *Cancer Res.*, 1952, v12, 251 (Abstr.).

10. Rose, F. L., Hendry, J. A., and Walpole, A. L., *Nature*, 1950, v165, 993.

11. Farber, S., Appleton, R., Downing, V., Heald, F., King, J., and Toch, R., *Cancer*, 1953, v6, 135.

12. Sykes, M. P., Karnosky, D. A., Philips, F. S., and Burchenal, J. H., *Cancer*, 1953, v6, 142.

13. Lewis, M. R., *Am. J. Cancer*, 1937, v29, 510.

14. Roffo, A. H., *Bol. Inst. Med. Exp. Estud. Cancer*, 1940, v17, 415.

Received May 4, 1953. P.S.E.B.M., 1953, v83.

Response to and Assay of Vitamin B_{12} by a Mutant of *Escherichia coli*.* (20382)

K. R. Johansson.

From the Department of Bacteriology and Immunology, University of Minnesota, Minneapolis.

The difficulties encountered in the assay of vit. B_{12} by strains of *Lactobacillus lactis* and *L. leichmannii* are well known(1-7). Since Davis and Mingioli(8) suggested that certain B_{12}-requiring mutants of *Escherichia coli* might be employed for the assay of this factor, several workers(9-11) developed an agar plate-disc assay of moderate sensitivity for B_{12} with one of these cultures. Others(12,13) have utilized *E. coli* 113-3 (strain designation of Davis and Mingioli) (8) for the assay of vit. B_{12} by measuring the response turbidimetrically.

It is the purpose of this report, an elaboration of a preliminary note(14), to describe a turbidimetric assay for B_{12} in which *E. coli* 113-3 is the test organism, and to present data concerning the influence of several factors upon the growth stimulus exerted by vit. B_{12} and methionine† upon this mutant.

Materials and methods. The mineral salts-citrate-glucose medium of Davis and Mingioli (8), modified by the addition of 0.05% sodium chloride, was adopted as the basal medium. The procedure was carried out in chemically-clean Pyrex test tubes (16 x 150 mm) in the conventional manner. Sequential quantities, 5 ml or less, of the standard solutions (from 2 to 70 $\mu\mu$g Merck's crystalline B_{12}‡ per ml, final concentration) or of a material being assayed, were added to a duplicate series of tubes, the volume adjusted to 5 ml with distilled water and 5 ml double strength basal medium added. The tubes were covered with aluminum caps, autoclaved at 120°C for 5 minutes and rapidly cooled. The inoculum consisted of one drop (0.05-0.07 ml) per tube of a 1:50 dilution of saline-washed cells of *E. coli* 113-3, previously grown for 18 hours at 30°C in the basal medium containing 15 μg L-methionine per ml. The stock culture was maintained by monthly transfer on slants of Difco's nutrient agar. Following inoculation, the tubes were incubated without agitation at 37°C for 16 hours, cooled and the amount of growth measured in a Klett-Summerson photoelectric colorimeter (blue filter). The readings of at least 4 tubes containing different amounts of test material were used in estimating the potency of samples assayed. Tissues were usually digested under toluene with trypsin at a pH of 8.0 and a temperature of 37°C for 24 hours prior to the assay. The response of *E. coli* 113-3 to methionine or

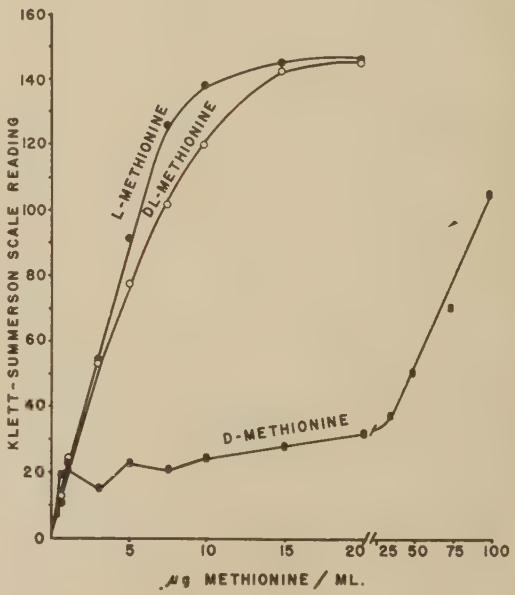


FIG. 1. Response of *Escherichia coli* 113-3 to methionine. Static incubation at 37°C in small tubes (16 × 150 mm) for 18 hr.

---

* Supported in part by research grant from the National Microbiological Institute of the National Institutes of Health, Public Health Service.

† This strain of *E. coli* responds to either methionine or vit. B_{12} in a medium otherwise free of organic growth factors. No other substance, except possibly cytochrome c(15), will replace its demand for these factors.

‡ Kindly furnished by Dr. Karl Folkers, Merck and Co., Rahway, N. J.

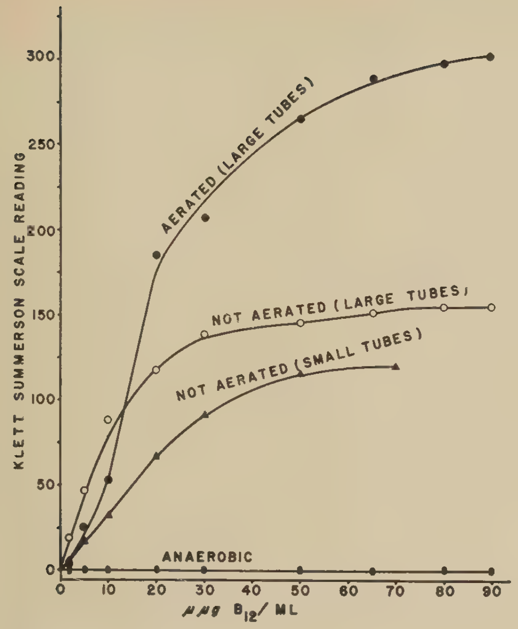


FIG. 2. Effects of degree of aerobiosis upon response of *Escherichia coli* 113-3 to vit. B_{12}. Test tubes used were 25 × 150 mm (large) and 16 × 150 mm (small).

B_{12} could be determined without autoclaving by employing usual aseptic methods with sterile glassware and reagents. For anaerobic incubation, aluminum pressure cookers, equipped with palladium-asbestos catalyzers and contained in an incubator, were filled with the inoculated tubes (25 x 150 mm), evacuated twice by means of a water pump and brought to atmospheric pressure with a 1:9 mixture of carbon dioxide and hydrogen.§

Results. Influence of several factors on response of Escherichia coli 113-3 to vit. B_{12}. It may be seen in Fig. 1 that this bacterium responded to L-methionine at concentrations of from <0.5 to >10.0, and to DL-methionine from <0.5 to 20.0 μg per ml. D-methionine was not appreciably stimulatory in concentrations of less than 25.0 μg per ml, although low concentrations (<1.0 μg per ml) were more stimulatory to growth than either the L or DL isomers.

The response of *E. coli* to vit. B_{12} was shown to vary with the degree of aerobiosis (Fig. 2). This effect, not surprising in view of the ability of B_{12} to stimulate the oxidative action of *E. coli* 113-3(15) and the influence of the redox potential upon the B_{12} requirement of *L. lactis*(3,6,16), has been noted by others (8,13). Thus, continuous shaking of graded levels of vit. B_{12} in large tubes during a 16-hour period resulted in markedly heavier growth than did static cultures in either the large or the small tubes, as did the greater surface area exposed in non-aerated large tubes than in small tubes. Strict anaerobiosis resulted in complete suppression of the growth stimulating property of B_{12} for this mutant. Likewise, L-methionine did not promote growth if the atmosphere were oxygen-free, although the presence of both B_{12} and methionine, at concentrations of greater than 100 μμg and 10 μg per ml, respectively, permitted submaximal growth under anaerobic conditions. The curve in Fig. 2 designated "not aerated (small tubes)" is representative of the usual conditions employed in this study and may be considered to be, therefore, a typical standard curve. Under these "standard" conditions, *E. coli* 113-3 responded linearly to B_{12} at concentrations of from <2.0 to approximately 20.0 μμg per ml while the maximal stimulating level was 75.0 μμg. On occasion, a response could be measured with 0.5 μμg, but 1.0 μμg invariably evoked a growth stimulation.

Cyanide has been shown to react with cobalamins resulting in a heat stable form analogous to cyanocobalamin(17,18), while certain reducing agents have been demonstrated to "protect" the heat labile forms of this vitamin(4,5,13,19,20). Table I shows that concentrations of potassium cyanide greater than 1.0 μg and of sodium thioglycolate or ascorbic acid greater than 10.0 and 5.0 μg per ml, respectively, suppressed the B_{12}-induced growth response of *E. coli*. If, however, the tests were made aseptically, cyanide at a level as low as 0.5 μg per ml was inhibitory, while thioglycolate was not inhibitory at a concentration as great as 500 μg per ml; ascorbic acid was just as inhibitory without as with the autoclaving treatment. At higher concentrations, B_{12} overcame the antagonistic action of cyanide and thioglycolate. It may be seen

---

§ An apparatus designed and built by Dr. D. W. Watson of this department.

TABLE I. Effect of Cyanide and Reducing Agents on Response of *E. coli* 113-3 to Vit. B_{12}.

B_{12} ($\mu\mu$g/ml)	KCN (μg/ml) 1	5	10	25	Na thioglycolate (μg/ml) 10	100	250	500	Ascorbic acid (μg/ml) 1	5	100	250	Control
5	16*	14	9	1	15	12	9	2	15	16	12	2	17
10	33	28	19	2	31	23	13	3	32	31	21	2	32
30	90	83	52	5	91	55	20	2	89	87	60	5	92
60	119	113	96	6	117	97	29	4	115	114	91	7	117

* All tubes autoclaved at 120°C 5 min. Growth expressed as scale reading of Klett-Summerson photoelectric colorimeter (avg of several trials). Zero settings made with uninoculated medium.

TABLE II. Effect of Autoclaving on Response of *E. coli* 113-3 to Vit. B_{12} and B_{12a}.

B_{12} or B_{12a} ($\mu\mu$g/ml)	B_{12} Autocl.*	Aseptic	B_{12a} Autocl.*	Aseptic
5	17	15	9	12
10	34	32	16	23
30	92	90	52	68
60	116	113	92	104
100	120	119	105	115

* See footnote, Table I.

TABLE III. Demonstration, with *E. coli* 113-3, of Action of Cyanide and Reducing Agents upon Vit. B_{12a}.

B_{12a} ($\mu\mu$g/ml)	KCN (1 μg/ml)	Na thioglycolate (5 μg/ml)	Ascorbic acid (5 μg/ml)	Control
5	17*	14	7	8
10	31	28	14	15
30	89	79	41	54
60	109	99	81	89
100	119	116	103	104

* See footnote, Table I.

(Table II) that vit. B_{12a},[‖] unlike B_{12}, lost activity (approximately 30%) upon autoclaving, unless low concentrations of cyanide and thioglycolate (Table III) were present. Moreover, cyanide caused conversion of B_{12a} to a cyanocobalamin nearly equivalent to vit. B_{12}, in terms of activity for the test organism. At the concentrations used, ascorbic acid was of no value in "protecting" vit. B_{12a} from heat inactivation. Vit. B_{12a}, when tested aseptically, was approximately 70% as active as B_{12} for this mutant (Table II); this is a favorable comparison with the 75% figure reported by Chiao and Peterson(13).

Assay of vit. B_{12}. Only those materials, *e.g.*, soy bean meal, which contain inappreciable quantities of B_{12} and are relatively rich in methionine were difficult, if not impossible, to assay. The calculated potency of B_{12} in certain materials was found to be influenced by the presence of cyanide or thioglycolate in the basal medium, presumably as a consequence of the presence of heat labile cobalamins. The results of a study with such a substance, a dried streptomyces fermentation concentrate,[¶] are assembled in Table IV. Note that, while the standard deviation for a single test was relatively low, a noticeable discrepancy between trials was encountered. The latter may be a reflection, to a large extent, of variations between the one-gram aliquots weighed for each determination. As expected, cyanide (1.0 μg per ml) and, to a lesser extent, thioglycolate (5.0 μg per ml) raised the calculated potency of B_{12} over that estimated in the unsupplemented basal medium. If the concentrate was assayed without autoclaving, the assay values were similar to those obtained by autoclaving in the presence of cyanide. Results with the latter methods were comparable to those obtained with *L. leichmannii* 4797 (method of Capps *et al.*(21), modified by the addition to the basal medium of 0.1% sodium thioglycolate, but not corrected for a response to desoxyribosides). The assay consistently accounted for all (98 to

---

[‖] Crystalline vit. B_{12a} furnished through generosity of Drs. G. E. Boxer and D. Hendlin, Merck and Co., Rahway, N. J. Although it had initially, $<$5.0% of other forms of this vitamin, it was found after several months by Dr. Boxer to contain approximately 18% cyanocobalamin. Unless contamination with cyanide occurred, this transformation is difficult to explain.

[¶] Kindly furnished by Dr. A. M. Hanson, Grain Processing Corp., Muscatine, Ia.

TABLE IV. Vit. B_{12} Assays of a Dried Streptomyces Fermentation Concentrate. Comparison between *Escherichia coli* 113-3 under Different Conditions and *Lactobacillus leichmannii* 4797.

	E. coli				
Trial No.	Autoclaved control	Aseptic control	+KCN (1.0 μg/ml)	+Na thioglycolate (5.0 μg/ml)	*L. leichmannii**
1	8.9 ± .32†	12.0 ± .40	12.4 ± .24	11.6 ± .46	—
2	9.7 ± .19	14.9 ± .08	16.0 ± .24	—	13.6 ± .28
3	8.0 ± .16	—	12.6 ± .34	—	13.5 ± .45
4	7.8 ± .24	—	—	10.0 ± .46	11.2 ± .68
Avg	8.60	13.45	13.67	10.80	12.77

* Method of Capps *et al.*(21) modified by addition of 0.1% sodium thioglycolate to basal medium.

† Average of at least 4 levels of test material, ± stand. dev. $\left(\sqrt{\frac{\Sigma x^2}{n-1}}\right)$, expressed as μg/g.

103%) of the B_{12} added in known amounts.

Discussion. It is clear that a number of factors affect the assay of vit. B_{12} with *E. coli* 113-3, *viz.*, 1) the degree of aerobiosis during incubation, 2) the presence of methionine (all 3 isomers) in the test substance, 3) the kinds of cobalamins present, 4) the nature and concentration of reducing agents in the medium, 5) the concentration of cyanide, if present, and 6) the employment of heat. Although aeration during incubation stimulated the response of the culture to B_{12}, the advantage, in terms of an assay, was no greater than with static incubation. The upper limit of response to B_{12} was higher with than without aeration but the sensitivity of *E. coli* 113-3 to small amounts of B_{12} was greater without agitation. Furthermore, agitation caused difficulty in reproducibility. Consequently, aeration, advocated by other workers(12,13), serves to complicate the assay without an improvement in precision or sensitivity. The dual response of *E. coli* 113-3 to B_{12} and methionine causes obvious difficulties. Nevertheless, most materials of animal origin lend themselves to this assay since the half-maximal response of *E. coli* 113-3 to vit. B_{12} and methionine under "standard" conditions was approximately 18 μμg and 2.5 μg per ml, respectively. When a methionine interference is suspected, such suspicion may be confirmed by applying a mild alkaline hydrolysis and assaying directly for methionine. Chiao and Peterson(13) report that if the ratio of methionine to B_{12} on a weight basis is less than 50,000, the former will not influence the specificity of the assay with this mutant. That D-methionine was increasingly stimulatory at high levels while supporting slight growth at lower concentrations is suggestive of a methionine isomerase in these cells.

Unfortunately, the different cobalamins with varying biochemical activities both in bacteria and animals continues to plague any biological assay for vit. B_{12}. While the rat responds equally well to B_{12} and B_{12a}(22), B_{12a} was 0.7 (0.4 with autoclaving treatment) as active toward *E. coli* 113-3 as B_{12}. Chiao and Peterson(13) found vit. B_{12f} to be 0.4 as active for this mutant as B_{12} while the former is inactive for the rat(23). Attempts have been made by numerous investigators to "protect" the heat labile forms of cobalamins by the incorporation of reducing agents in the medium. This study indicates that the nature and concentration of reducing agents are critical. Ascorbic acid was of no value in protecting vit. B_{12a} from partial heat inactivation while sodium thioglycolate was useful in this respect. However, relatively high concentrations of these reducing agents completely destroyed the growth stimulating property of B_{12} and B_{12a}, an observation compatible with earlier reports(24,25). A further complication of reducing agents is the report(26) that thiomalic and thioglycolic acids convert vit. B_{12} to an analogue more active than B_{12} toward *L. leichmannii*. If such a substance was formed during our study, it was no more active—indeed, probably less active—for *E. coli* than vit. B_{12}, *per se*. Apparently, the optimal concentration of thioglycolate depends on the composition of the basal medium. For example, Burkholder(12) used 0.1 mg sodium thioglycolate per ml in his *E. coli* 113-3 assay medium, an amount shown by our study to suppress this organism's growth to a significant extent. Burkholder's medium was, however, complex, containing more glucose** and 7 amino acids.

The value of including cyanide in the medium when assaying for vit. B_{12} with *L. lactis* and *L. leichmannii* has been established(27, 28). In this work, cyanide appeared to convert B_{12a} to a cyanocobalamin equivalent in activity for *E. coli* 113-3 to that of B_{12}. Inasmuch as this organism is very sensitive to cyanide, the concentration used must be below a level of 5 μg per ml basal medium. The greater inhibition by cyanide of the response of *E. coli* to B_{12} or B_{12a} when tested aseptically than when determined following autoclaving was provoking. Perhaps at higher temperatures cyanide is altered, or a product results from the reaction of cyanide with cobalamins which is inactive for the test organism. Until the significance of the various cobalamins is clarified, it would seem desirable to include cyanide in the basal medium at some level less than 5 μg per ml. However, if the assay is to be made without autoclaving, cyanide should be omitted from the medium. Furthermore, there is good agreement when natural substances, *e.g.* streptomyces concentrate, liver, feces, etc., are assayed simultaneously by autoclaving in the presence of CN^-, and by asepsis without CN^-.

Although this mutant is used by a number of laboratories for the assay of vit. B_{12} by means of the agar plate-disc technic(9,10), or modifications thereof, a few trials in this laboratory found the plate method to have less sensitivity than the more tedious turbidimetric procedures. The assay procedure reported herein compares favorably to the similar one of Chiao and Peterson(13) but avoids, without reduction in precision, both agitation during incubation and pre-treatment with potassium cyanide. The turbidimetric method of Burkholder(12) appears to be less sensitive with no greater a range of linear response to vit. B_{12}. This method of assay for B_{12} has numerous advantages over those requiring a *Lactobacillus* spp., not the least of which is its relative simplicity.

Summary. A mutant organism, *Escherichia coli* 113-3, which responds to vit. B_{12} or methionine in a mineral salts-citrate-glucose medium, was adopted successfully for the assay of vit. B_{12}. The effects of the degree of aerobiosis, and of potassium cyanide and two reducing agents, upon the response of this bacterium to vit. B_{12} and B_{12a} are described.

** To determine whether or not a constituent in the medium augmented destructive action of heat on vit. B_{12a}, it was noted that less destruction occurred if glucose was omitted from the medium. The slight degree of heat inactivation (5 min. at 120°C) of B_{12a} in distilled water was the same as that which occurred in the glucose-free medium.

1. Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, v175, 475.

2. Shorb, M. S., and Briggs, G. M., *J. Biol. Chem.*, 1948, v176, 1463.

3. Caswell, M. C., Koditschek, L. K., and Hendlin, D., *J. Biol. Chem.*, 1949, v180, 125.

4. Hoffman, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C., and Jukes, T. H., *J. Biol. Chem.*, 1949, v181, 635.

5. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *J. Biol. Chem.*, 1950, v184, 211.

6. Cuthbertson, W. F. J., and Lloyd, J. R., *J. Gen. Microbiol.*, 1951, v5, 416.

7. Zygmunt, W. A., *J. Bact.*, 1952, v63, 821.

8. Davis, B. D., and Mingioli, E. S., *J. Bact.*, 1950, v60, 17.

9. Bessell, C. J., Harrison, E., and Lees, K. A., *Chem. Ind.*, 1950, 561.

10. Harrison, E., Lees, K. A., and Wood, F., *Analyst*, 1951, v76, 696.

11. Williams, W. L., Esposito, R. G., and Pierce, J. V., *Fed. Proc.*, 1952, v11, 458.

12. Burkholder, P. R., *Science*, 1951, v114, 459.

13. Chiao, J. S., and Peterson, W. H., *Applied Microbiol.*, 1953, v1, 42.

14. Johansson, K. R., *Bact. Proc.*, 1951, 146.

15. Oginsky, E. L., Smith, P. H., Tonhazy, N. E., Umbreit, W. W., Lichstein, H. C., and Carson, S. F., *J. Bact.*, 1951, v61, 581.

16. Koditschek, L. K., Hendlin, D., and Woodruff, H. B., *J. Biol. Chem.*, 1949, v179, 1093.

17. Kaczka, E. A., Wolf, D. E., Kuehl, F. A., Jr., and Folkers, K., *Science*, 1950, v112, 354.

18. Veer, W. L. C., Edelhausen, J. H., Wijmenga,

H. G., and Lens, J., *Biochim. Biophys. Act.*, 1950, v6, 225.

19. Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, v8, 257.

20. Thompson, H. T., Dietrich, L. S., and Elvehjem, C. A., *J. Biol. Chem.*, 1950, v184, 175.

21. Capps, B. F., Hobbs, N. L., and Fox, S. H., *J. Biol. Chem.*, 1949, v178, 517.

22. Frost, D. V., Fricke, H. H., and Spruth, H. C., *J. Nutrition*, 1953, v49, 107.

23. Lewis, U. J., Tappan, D. V., and Elvehjem, C. A., *J. Biol. Chem.*, 1952, v199, 517.

24. Lang, C. A., and Chow, B. F., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 39.

25. Hendlin, D., and Soars, M. H., *J. Biol. Chem.*, 1951, v188, 603.

26. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 806.

27. Cooperman, J. M., Drucker, R., and Tabenkin, B., *J. Biol. Chem.*, 1951, v191, 135.

28. Soars, M. H., and Hendlin, D., *J. Bact.*, 1951, v62, 15.

Received May 5, 1953. P.S.E.B.M., 1953, v83.

Effect of Injecting Platelet Extract Intravenously.*† (20383)

ERNST EPSTEIN AND ARMAND J. QUICK.
From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wis.

It is now fairly widely accepted that platelets do not contain any significant amount of preformed thromboplastin, but supply a factor which reacts with a plasma constituent, thromboplastinogen, to form active thromboplastin (1). Recent studies indicate that before this reaction can occur, thromboplastinogen must become activated either by contact with a foreign surface such as glass(2) or by the addition of thrombin(3). Since circulating blood probably contains no activated thromboplastinogen, the injection of a platelet extract should not cause intravascular clotting nor have any pronounced effect on the blood. The present investigation was undertaken to test the correctness of this conclusion. As a control, the effect of injecting tissue extract containing active thromboplastin was also studied.

Methods. Platelet extract from rabbit blood was prepared according to the method previously described(2). The procedure consists essentially in obtaining platelets by differential centrifugation, and then causing disintegration by repeated freezing and thawing while suspended in distilled water. This procedure is very effective for disintegrating platelets as revealed by phase contrast microscopy and by the loss of all power to bring about clot retraction. *Rabbit brain extract* was prepared by triturating fresh brain tissue, cleared of blood vessels, with distilled water, then subjecting it to freezing and thawing similar to the procedure used for platelets. The emulsion was centrifuged for 5 minutes at 1000 rpm to remove coarse particles and then was allowed to remain at room temperature for 3 hours to assure inactivation of any adenosine triphosphate(4). Both the platelet extract and the brain extract were adjusted to a sodium chloride concentration of 0.85%. The *fibrinogen concentration* was determined by the method previously described(5) except that instead of employing the Folin Ciocalteu reagent, the fibrin digested in sodium hydroxide was estimated directly by determining the optical density at 280 μ. The *prothrombin time* and prothrombin consumption time were determined by the methods previously described(5). Platelets were counted directly in a hemacytometer using 3.8% sodium citrate solution as diluent. All injections were made into the marginal vein of the rabbit ear and were given at the rate of about 3-5 cc per minute. Blood samples for analysis were taken from the central artery of the ear.

Results. When a potent extract of platelets in an amount equivalent to as high as 210%

---

* The authors acknowledge the valuable assistance of Dr. Edward Barta of the Department of Pathology who furnished most of the animals used.

† This investigation was supported by a grant from the Division of Research Grants, National Institute of Health, USPHS.

TABLE I. Effect of Injecting Platelet Extract Intravenously into 6 Rabbits.

Wt of rabbit (kilos)	No. of platelets inj. as extract	Calculated equivalent % of platelet in circulation	Fibrinogen conc. (mg/100 cc of plasma)				
				After platelet extract inj.			
			Before	5 min.	15 min.	45 min.	4 hr
4.0	7.2×10^{10}	25	176	168	180	—	—
1.6	10.3×10^{10}	210	258	—	249	—	244
3.4	4.2×10^{10}	42	180	182	—	190	—
4.0	28.0×10^{10}	30	213	202	—	219	—
2.7	7.8×10^{10}	70	277	289	—	270	—
3.4	5.3×10^{10}	142	166	168	—	173	—

Prothrombin time in all animals remained normal (6 sec.), prothrombin consumption time of whole blood occasionally showed slight but insignificant variations. Platelet count always decreased, but rarely below lower range of normal.

Platelet extract inj. into rabbit #6 contained 234 mg of platelet solids in 12 ml of extract. Two weeks later a brain tissue extract containing 52 mg of brain solids in 12 ml of extract was inj. at same rate. The rabbit died in less than 3 min. after the end of the inj. Autopsy showed massive pulmonary embolism.

of the number of platelets in circulation was injected intravenously into rabbits, no striking systemic effects were noted, the prothrombin time and the prothrombin consumption times were not significantly altered and the fibrinogen concentration remained unchanged (Table I). Thus, the injection of a platelet extract containing 234 mg of platelet solids was entirely innocuous, yet the injection of rabbit brain extract containing 52 mg of brain solids into the same animal employing the same technic, killed the animal in 3 minutes by causing a massive pulmonary embolism. The ineffectiveness of platelet extract *in vivo* cannot be attributed to lack of potency since the material employed brought about a marked consumption of prothrombin when added to normal deplateletized rabbit plasma and decreased the clotting time of recalcified highly-centrifuged plasma when the reaction was carried out in glass test tubes (Table II).

TABLE II. Potency of Platelet Extract as Estimated by Prothrombin Consumption Time and Clotting Time of Highly-Centrifuged, Recalcified Citrated Rabbit Plasma.

	Conc. of platelet extract, %				
	0	1	3	10	100
Clotting time of recalcified plasma (sec.)	1600	520	180	145	125
Prothrombin consumption time 1 hr after clotting (sec.)	9	14	27	41	63

To 1 ml of citrated plasma were added 0.1 ml of platelet extract and 0.1 ml of 0.25 M $CaCl_2$. After mixing, the tube was incubated at 37°C. Platelet extract used was an aliquot of that inj. into rabbit #6 in Table I. Undiluted platelet extract (100%) contained approximately 20 mg of platelet solids/ml.

The results reported in this study clearly suggest that the products from mechanically disintegrated platelets are inert in circulating blood. This is in accord with our recent findings that the platelet factor can act only after thromboplastinogen has been activated. The present findings fail to support the theory of Brinkhous(6) that "normal plasma contains a factor necessary for thromboplastin liberation from formed elements of the blood, presumably by platelet lysis." In conformity with our previous findings(2), it is unlikely that the clotting agent in platelets is an enzyme as one of us had postulated(5). It is probable that intravascular clotting is initiated only if either thrombin or preformed thromboplastin such as occurs in tissues gains entrance into the blood stream.

Summary. The injection of a potent extract of rabbit platelets into normal rabbits fails to produce any demonstrable effect on the coagulation system and does not induce intravascular clotting.

---

1. Quick, A. J., *Am. J. Med. Sci.*, 1947, v214, 272.
2. Quick, A. J., and Epstein, E., *J. Applied Physiol.*, 1952, v4, 840.
3. Quick, A. J., Hussey, C. V., and Epstein, E., *Am. J. Physiol.*, in press.
4. Green, H. N., and Stoner, H. B., *Brit. J. Exp. Path.*, 1947, v28, 189.
5. Quick, A. J., *The Physiology and Pathology of Hemostasis*, Philadelphia, Lea and Febiger, 1951.
6. Brinkhous, K. M., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 117.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

Protamine-like Property of Compounds 48/80 and Stilbamidine and Their Action on Mast Cells.* (20384)

I. Mota, W. T. Beraldo, and L. C. U. Junqueira.

From the Laboratory for Cell Physiology and Department of Physiology, Faculdade de Medicina, Universidade de S. Paulo, Brasil.

Compound 48/80, obtained from the condensation of p-methoxyphenethyl-methylamine with formaldehyde(1), is the most active histamine liberator so far described(2). Stilbamidine, a trypanocidal diamine(3) was also observed to be a histamine liberator(4). The present paper deals with the effect of these compounds on tissue mast cells and their action on the anti-coagulant property of heparin and allied substances.

Material and methods. The effect of these compounds on the mast cells of male Wistar rats (weighing 200-300 g) was studied both *in vivo* and *in vitro*. In the experiments *in vivo* the animals under light ether anesthesia were injected intravenously with 5 mg per kg of each of the studied compounds, diluted in 1 ml saline (0.9% NaCl). Both compounds produced a severe histamine shock with death of the animal in 5 to 8 minutes. Ten rats injected with one ml of saline and sacrificed with ether were used as controls for similar groups injected with Compound 48/80 or Stilbamidine. To avoid artifacts due to common histologic technic, mesentery mast cells were fixed by aortic perfusion. Ten minutes later the abdominal cavity was opened and the mesentery fixed for 12 hours more with Bouin's fluid or Holmgren and Wilander's lead acetate(5) modified by addition of 10% formaldehyde. The same results were obtained with either fixative. Mast cells were observed after staining with 1% toluidine blue or unstained with phase contrast microscopy. In all rats injected with Compounds 48/80 and Stilbamidine, coagulation time was determined by the Lee-White method(6). The experiments *in vitro* were performed by immersing fragments of mesentery into saline containing different concentrations (from 1/1000 to 1/20000) of either Compound 48/80 or Stilbamidine, for 5 minutes at room temperature (25°C). Controls were immersed in saline for the same period. These fragments were then fixed and stained by transferring them to a 10% formaldehyde solution containing 1% toluidine blue. To observe the protamine-like action of Compound 48/80 and Stilbamidine, different quantities of these substances were dissolved in saline and mixed with whole blood containing heparin (Vitrum), Liquoid (Roche) or cellulose sulphate and the coagulation time was determined.

Results. In the experiments *in vivo,* the injection of Compound 48/80 or Stilbamidine always produced bursting of mesentery mast cells with extrusion of granules (Fig. 1). This was more evident and always present in perivascularly located mast cells. No change could be observed in the coagulation time of the injected animals.

Both Compound 48/80 and Stilbamidine promoted a generalized disruption and granule liberation of mesentery mast cells *in vitro,* with no preference for perivascular cells. Heparin when previously added to solutions of both compounds inhibited the disruption of mast cells. Due mainly to traction of the mesentery during dissection, bursting of mast cells could be occasionally observed in the controls, but this was easily recognized as an artifact. The bursting effect with Compound 48/80 could be observed under the phase contrast microscope as a rapid bubbling of the cell surface during a few seconds.

Tables I and II show the results obtained in a typical experiment studying the anti-heparin effect of Compound 48/80 and Stilbamidine. Inspection of these tables demonstrates an anti-heparin effect of these compounds, and with higher concentration an increase in the coagulation time as well.

Discussion. The alteration of mast cells in peptone and anaphylactic shock has been related to heparin liberation(7,8). Although

---

* This work was partially aided by a grant from the Conselho Nacional de Pesquizas.

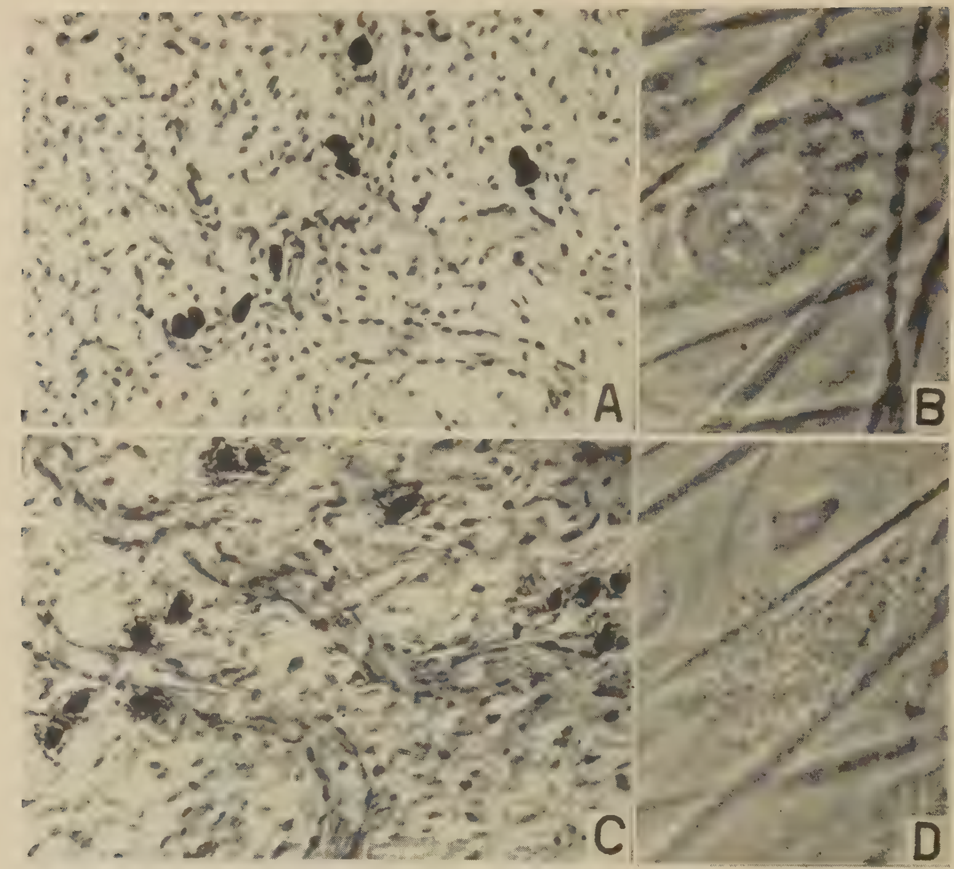


FIG. 1. Mast cells of rat mesentery. A and C toluidine blue stained (×400). B and D phase contrast (×1300). A and B from control animal. C and D compound 48/80 injected animal.

these results were confirmed in the dog, in the rat the disruption of mast cells evoked by either anaphylactic, peptone shock(9), Compound 48/80 or Stilbamidine, does not seem to be accompanied by heparin liberation if judged by blood coagulation time. In the case of the last 2 substances this phenomenon might be related to their anti-heparin effect. However the fact that in peptone and anaphylactic shock no substance with anti-heparin effect exists, might suggest that heparin in the rat mast cell is bound in a more stable linkage than in the dog mast cell. It is interesting to point out here the difficulty met with in the histologic fixation of the dog mast cell as compared to the rat mast cell.

The striking positive correlation observed between the mast cell content of a tissue and its concentration of histamine(10) suggests that mast cells contain histamine. Riley(11), also observed that Stilbamidine disrupts mast cells *in vivo*. The fact that mast cell disruption occurs in conditions in which histamine liberation is observed, as in anaphylactic and peptone shock(7-9), coupled with our observations that Compound 48/80 and Stilbamidine cause the break-up of mast cells *in vivo* and *in vitro,* lend support to the hypothesis that histamine arises from mast cells. If this is true, mast cells probably would be one of, but not the only source of tissue histamine, since it is known that histamine may be obtained *in vitro* from tissue free of mast cells such as blood(12). The way histamine may be bound to the mast cell's cytoplasm and its relation to heparin, constitutes a challenging problem.

The observations that Compound 48/80 or Stilbamidine increase coagulation time and the fact that these compounds counteract the anti-

TABLE I. Action of Compound 48/80 on Anti-Coagulant Property of Heparin, Liquoid and Cellulose Sulphate.

C. 48/80, µg/ml final vol	Coagulation time of blood:		
	+ heparin + C. 48/80	+ Liquoid + C. 48/80	+ cellulose sulphate + C. 48/80
0	>20 hr	>20 hr	>20 hr
20	7′00″	4′30″	>20 hr
40	3′45″	5′00″	>20 hr
60	5′30″	5′10″	20′10″
80	5′50″	7′10″	8′20″
100	18′10″	12′30″	7′50″
200	27′20″	18′20″	8′10″
500	>20 hr	40′15″	>20 hr

Blood plus heparin 5 U/ml. Blood plus Liquoid or cellulose sulphate 100 µg/ml. 0.5 ml of rat's blood was added to each test tube. The final vol (1 ml) was completed with saline.

TABLE II. Action of Stilbamidine on Anti-Coagulant Property of Heparin, Liquoid and Cellulose Sulphate.

Stilbamidine, µg/ml of final vol	Coagulation time of blood:		
	+heparin +Stilbamidine	+Liquoid +Stilbamidine	+cellulose sulphate +Stilbamidine
0	>20 hr	>20 hr	>20 hr
20	9′50″	40′00″	>20 hr
40	9′50″	10′00″	20′10″
60	13′30″	8′10″	14′30″
80	20′40″	15′00″	15′35″
100	30′50″	40′20″	30′10″
200	>20 hr	>20 hr	>20 hr
500	>20 hr	>20 hr	>20 hr

Blood plus heparin 5 U/ml. Blood plus Liquoid or cellulose sulphate 100 µg/ml. 0.5 ml of rat's blood was added to each test tube. The final vol (1 ml) was completed with saline.

coagulant action of heparin and allied substances, strongly suggest a protamine-like action of these compounds. The addition of Compound 48/80 or Stilbamidine to a solution of heparin, Liquoid or cellulose sulphate, causes a precipitate(9) and one might suppose that these substances act like protamines with the formation of insoluble complexes which remove heparin from the system.

Summary. Compound 48/80 and Stilbamidine produce both *in vivo* and *in vitro* bursting of rat's mast cells. This phenomenon was observed with phase contrast microscopy as a rapid bubbling of the cell surface. These compounds also possess a protamine-like effect. The possible significance of these results is discussed.

The authors are grateful to Dr. E. J. de Beer, the Wellcome Research Laboratories, Tuckahoe, N. Y. for generous supplies of Compound 48/80 and to Dr. A. Rothschild from Instituto Biológico, Brasil, who kindly supplied the cellulose sulphate.

---

1. Baltzly, R., Buck, J. S., de Beer, E. J., and Webb, F. J., *J. Am. Chem. Soc.,* 1949, v71, 1301.

2. Paton, W. D. M., *Brit. J. Pharmacol. Chemotherapy,* 1951, v6, 499.

3. Lourie, E. M., and Yorke, W., *Ann. Trop. Med. Parasit.,* 1939, v33, 289.

4. MacIntosch, F. C., and Paton, W. D. M., *J. Physiol.,* 1949, v109, 190.

5. Holmgren, H., and Wilander, O., *Z. f. mikr. Anat. Forsch.,* 1937, v42, 242.

6. Wintrobe, M. M., *Clinical Hematology,* 1947, Lea & Febiger, Philadelphia.

7. Wilander, O., *Skandinav. Arch. f. Physiol.,* 1939, v81, Supp. XV.

8. Jaques, L. S., and Waters, E. T., *J. Physiol.,* 1941, v99, 454.

9. Mota, I., and Beraldo, W. T., unpublished results.

10. Riley, J. F., and West, G. B., *J. Physiol.,* 1952, v117, 72 P.

11. Riley, J. F., *Proc. Scot. Exp. Med.,* Dundee Meeting, 9 Feb. 1952.

12. Katz, G., *Science,* 1940, v91, 221.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

Feeding Rats Tissues from Lambs and Butterfat from Cows that Consumed DDT-Dusted Alfalfa Hay.* (20385)

D. A. Greenwood, L. E. Harris, C. Biddulph, G. Q. Bateman, W. Binns,
M. L. Miner, J. R. Harris, F. Mangelson, and L. L. Madsen.

From the Utah Agricultural Experiment Station, Logan, Utah.

Investigations relating to the toxicity of DDT have been reviewed elsewhere(1-3). Previous studies by this station have been concerned with effects of feeding DDT-dusted alfalfa hay to fattening lambs(4,5), dairy cows(6), chickens(7,8), and swine(9).

The purpose of this paper is to report studies on the effects of feeding rats tissues from lambs and butterfat from cows that consumed DDT-dusted alfalfa hay.

Materials and methods. Alfalfa was dusted with technical DDT for control of insects at the following levels: no DDT, 0.5, 1, 2, and 4 lb. per acre as described previously(4). The hay thus treated was harvested and fed to 32 lambs for 120 days(5) and to 16 dairy cows for 113 days(6). An additional 32 lambs were fed DDT in capsules at levels of 0, 50, 100, and 200 ppm. of hay consumed(5). Following slaughter, the kidney and mesenteric fat and the boneless leg of each lamb were incorporated separately into diets (Table I). Food consumption was recorded. Milk obtained from cows that had consumed hay dusted with different levels of DDT(6) was separated, the cream churned, and the butterfat incorporated in the experimental diets as shown in Table I. The lamb fat and lamb leg diets were each fed to 4 weanling male and 4 weanling female rats (Tables II, III, and IV). The rats were housed in individual cages and weighed at 2-week intervals. The diets containing the lamb fat were fed for 16 weeks, those containing lamb leg for 12 weeks. The number of rats fed butterfat containing different levels of DDT is given in Table IV. They were fed for 16 weeks and then sacrificed and an autopsy made on each animal. Samples of liver and kidney were taken for histological study, and DDT determinations were made on the abdominal fat. The DDT content of the tissues and butterfat was determined by the colorimetric method of Schechter *et al.*(10,11) as modified by Harris *et al.*(4). The amount of DDT residue on the hay was determined by the total chloride method of Umhoefer(12).

Results and discussion. There were no significant differences in the growth of rats fed lamb fat and lamb leg containing the lowest and highest levels of DDT, or between control and experimental rats. Food consumption was essentially the same for all rats.

The average DDT residue on hay, lamb fat and leg, and the storage in rats fed lamb fat and leg are summarized in Tables II and III. The values for DDT in the rat fat varied from 10 to 144 ppm. Rats fed lamb leg stored higher levels of DDT than those fed lamb fat, even though the lamb leg contained less DDT and was fed for 12 weeks, whereas the diets containing lamb fat were fed for 16 weeks. The greater storage of DDT following intake of a smaller amount of insecticide in the diet is in essential agreement with the data previously reported(4) in which storage of DDT was greater in lambs fed DDT-dusted hay than in lambs fed a greater amount of DDT in capsules.

The storage of DDT in the fat of the rats fed butterfat is shown in Table IV. There was no statistically significant difference in the growth of rats fed butterfat containing the lowest and highest levels of DDT or between control and experimental rats. All of the rats in the above experiments gained at the same rate as rats fed the stock diet.

It should be noted that the fat of rats fed the control diets and undusted hay contained DDT. The exact cause of this is not known. A possible explanation for the accumulation of DDT in the fat of control animals is that DDT contamination occurred by way of dust

---

* Research supported in part by a research grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service, and published with the approval of the director of the Utah Agri. Exp. Station.

TABLE I. Composition of Rat Diets.

Ingredients	Dry matter of ingredients, %	Lamb fat as fed, %	Lamb leg as fed, %	Butterfat as fed, %
Lamb kidney and mesenteric fat	86.9	22	—	—
Lamb leg	38	—	65	—
Butterfat	83.5	—	—	22
Casein	93	22	—	22
Salt mixture*	95.7	4	2.4	4
Sodium chloride	100	1	—	1
Wheat germ oil	100	0.5	—	0.5
Liver concentration 1-20†	97.3	2	—	2
Brewers yeast	94.7	5	4	5
Dextrinized starch	92.6	43.5	28.1	43.5
Cod-liver oil‡	100	—	.5	—
Total		100	100	100
Dry matter		91.5	59.9	90
Ether extract		19.6	10.7	16.9
Protein		23.1	13.7	21.9

* Ingredients in salt mixture in %: NaCl 27.5, K_2HPO_4 25.85, $Ca(H_2PO_4)_2 \cdot H_2O$ 21.65, $CaCO_3$ 18.515, $MgCO_3$ 4.6, $FeC_6H_5O_7 \cdot 3H_2O$ 1.8, $CuSO_4 \cdot 5H_2O$ .02, $MnSO_4$ .035, $K_2Al_2\ (SO_4)_4$ .24, H_2O .01, KI .005, $CoCl_2 \cdot 6H_2O$ .01, $ZnCO_3$ .005, NaF .0001.

† Obtained from Wilson Laboratories, Chicago, Ill.

‡ Contained 3000 I. U. vit. A and 400 A. O. A. C. chick units vit. D/g.

TABLE II. DDT Content of Rats Fed Lamb Fat and Leg from Lambs That Had Consumed DDT-Dusted Alfalfa Hay (8 Rats in Each Series).

DDT		DDT			DDT		
App./acre, lb	Residue on hay, ppm.	In lamb fat, ppm.	In rat diet (dry basis), ppm.	In rat abdominal fat, ppm.	In lamb leg, ppm.	In rat diet (dry basis), ppm.	In rat abdominal fat, ppm.
0	0	2	.5	14	.0	.1	12
1	15	16	3.8	49	.35	.5	81
2	22	18	5.3	60	.64	.7	83
4	42	44	10.1	95	1.15	1.0	101

TABLE III. DDT Content of Rats Fed Lamb Fat and Leg from Lambs That Had Consumed DDT in Capsules (8 Rats in Each Series).

DDT fed in capsules to sheep, ppm. of hay	DDT			DDT		
	In lamb fat, ppm.	In rat diet (dry basis), ppm.	In rat abdominal fat, ppm.	In lamb leg fed to rats, ppm.	In rat diet (dry basis), ppm.	In rat abdominal fat, ppm.
0	.0	.1	10	.17	.3	38
50	19.6	4.7	67	.61	.7	90
100	32.1	7.7	95	.60	.7	93
200	73.4	17.6	100	1.56	1.8	144

from experimental diets containing DDT. Laug *et al.*(3) also found that the fat of control rats housed in the same and different rooms contained DDT, demonstrating the avidity with which adipose tissue stores DDT.

No symptoms of DDT poisoning or histological changes in liver or kidney were observed in any of the rats under the conditions of these experiments.

Laug *et al.*(3) reported hepatic cell alterations after feeding diets containing as little as 5 ppm. DDT but did not indicate the exact length of DDT feeding required to produce these changes. Although these experiments differ in many details from those conducted on rats by Deichmann *et al.*(2), the results obtained in the phases of the two studies that can be compared are in substantial agreement.

TABLE IV. DDT Content of Rats Fed Butter from Cows That Had Consumed DDT-Dusted Alfalfa Hay.

		DDT				
No. of rats*	No. of cows	Applied/acre,† lb	Residue on hay, ppm.	In butter-fat, ppm.	In rat diet (dry basis), ppm.	In rat abdominal fat, ppm.
16	4	.0	0	0	.1	12
8	2	.5	9	13	3	46
16	4	1.0	13	42	10	69
16	4	2.0	26	70	17	107
8	2	4.0	42	123‡	30	188

* Equal number of males and females.

† The .0, 1 and 2 lb levels of DDT applied in 1948 and 1949. The 0.5 lb level in 1949, and 4 lb in 1948.

‡ Estimated value.

There is a need for additional chronic studies on the toxicity of DDT and other insecticides in diets containing varying levels of fat and protein.

Summary. Growing rats were fed tissues from lambs or butterfat from cows that had consumed DDT-dusted alfalfa hay as part of their diets for 12 to 16 weeks. The concentration of DDT varied from .1 to 30 ppm. on a dry basis. No significant differences in food consumption, rate of growth, or tissue changes could be detected by gross and microscopic examination in the rats fed the highest and lowest levels of DDT. High concentrations of DDT in the diet of rats gave the greatest storage of it in their body fat.

---

1. Leary, J. C., Fishbein, W. I., and Salter, L. C., *DDT and the Insect Problem,* McGraw-Hill Co., New York, 1946.

2. Deichmann, W. B., Witherup, S., Kitzmiller, K. V., and Heyroth, F. F., *Toxicity of DDT,* Univ. of Cincinnati, F. F. Kettering Laboratory, 1950.

3. Laug, E. P., Nelson, A. A., Fitzhugh, O. G., and Kunze, F. M., *J. Pharm. Exp. Therap.,* 1950, v98, 268.

4. Harris, J. R., Biddulph, C., Greenwood, D. A., Harris, L. E., Bryson, M. J., Binns, W., Miner, M. L., and Madsen, L. L., *Arch. Biochem.,* 1949, v21, 370.

5. Harris, L. E., Myint, T., Biddulph, C., Greenwood, D. A., Binns, W., Miner, M. L., and Madsen, L. L., *J. Animal Sci.,* 1951, v10, 581.

6. Biddulph, C., Bateman, G. Q., Bryson, M. J., Harris, J. R., Greenwood, D. A., Binns, W., Miner, M. L., Harris, L. E., and Madsen, L. L., *Advances in Chemistry,* Series I, 1950, 237.

7. Bryson, M. J., Draper, C. I., Harris, J. R., Biddulph, C., Greenwood, D. A., Harris, L. E., Binns, W., Miner, M. L., and Madsen, L. L., *Advances in Chemistry,* Series I, 1950, 232.

8. Draper, C. I., Harris, J. R., Greenwood, D. A., Biddulph, C., Harris, L. E., Mangelson, F., Binns, W., and Miner, M. L., *Poultry Sci.,* 1952, v31, 388.

9. Harris, L. E., Harris, J. R., Mangelson, F. L., Greenwood, D. A., Biddulph, C., Binns, W., and Miner, M. L., Unpublished data.

10. Schecter, M. S., Porgorelskin, M. A., and Haller, H. L., *Anal. Chem.,* 1947, v19, 51.

11. Soloway, S. B., Hayes, R. A., and Haller, H. L., *Ind. Eng. Chem.,* Anal. Ed., 1945, v17, 704.

12. Umhoefer, R. R., *Ind. Eng. Chem.,* Anal. Ed., 1943, v15, 383.

Received May 12, 1953. P.S.E.B.M., 1953, v83.

A Modified Method for Determination of Hydroxyproline.* (20386)

Charles J. Martin and A. E. Axelrod. (Introduced by Alan R. Moritz)

From the Institute of Pathology and the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, O.

During the course of investigations on the effects of thermal exposure upon rat skin and tail-tendon(1), we had occasion to utilize the method of Neuman and Logan for the determination of hydroxyproline(2) as a measure of the collagen content(3). In the application of this method considerable difficulty was experienced in obtaining reproducible, linear standard curves. Frequently, the standard curves would display the form of an exponential function, making the selection of suitable aliquots of an unknown troublesome. Consequently, the following study was undertaken in an effort to establish conditions which would consistently result in a linear proportionality between light absorption and known concentrations of hydroxyproline.

Experimental. The colorimetric method of Neuman and Logan(2) for the determination of hydroxyproline consists of the following steps: a) oxidation of the hydroxyproline with peroxide in the presence of alkali and copper sulfate; b) removal of the excess peroxide by heating at 80°C for 5 minutes; and c) color development with p-dimethylaminobenzaldehyde† in acid solution. If the excess peroxide is not removed, the color intensity is considerably decreased. It was felt that the erratic results obtained in this laboratory might be related to the step involving removal of the excess peroxide. However, variations in temperature between 70° and 80°C, and in the heating periods from two to ten minutes failed to yield a more consistent response. If the heating step was replaced by the addition of small amounts of ferrous sulfate to remove peroxide, a linear standard curve was obtained in the range of zero to 25 μg of hydroxyproline. In our procedure 0.1 ml of M/20 ferrous sulfate, containing 0.5 ml concentrated sulfuric acid per 100 ml, is added to each tube and shaken for 6 minutes. The concentration of the added ferrous sulfate solution could be varied from M/100 to M/10 with no change in color intensity. A qualitative test for peroxide, dependent on complex formation with dilute dichromate in acid solution, showed that the ferrous sulfate was efficacious in removing the excess peroxide after oxidation of the hydroxyproline.

In Fig. 1, the relationship between optical density at 550 mμ (determined in the Coleman Universal Spectrophotometer) and concentration of hydroxyproline is shown as determined by our modified procedure.

Prior to applying our modified method to the determination of hydroxyproline in collagenous tissue, we have found it expedient to make several changes in manipulative meth-

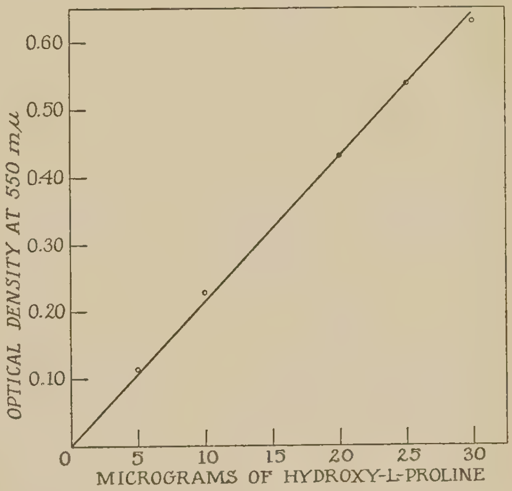


FIG. 1. Relation between color intensity and concentration of hydroxy-L-proline.

---

* This work performed under contract between the U. S. Atomic Energy Commission and Western Reserve University.

† We have found that several commercial samples of p-dimethylamino-benzaldehyde have proved refractory to purification by the method of Adams and Coleman(4) even when repeated 3 times and followed by crystallization from warm aqueous ethanol. Recrystallization from other solvents was also ineffectual. It was subsequently found that a product from the Matheson Co. could be used without purification.

odology. These included: a) the use of 2 ml aliquots of standard hydroxyproline solutions or of unknown rather than one ml aliquots; b) the addition of the copper sulfate and sodium hydroxide solutions in a single step (reagents mixed immediately before use) rather than separately; c) the addition of the color reagent jointly with the sulfuric acid; and d) the increase in the normality of the sulfuric acid from 3N to 4N to compensate for the increase in volume occasioned by doubling the volume of the test solution employed. An investigation of the reagent concentrations used in Neuman's and Logan's method indicated that they were present in optimal concentration for maximal color yield and, with the exception of changing the concentration of p-dimethylaminobenzaldehyde from 5 to 4%, the original reagent concentrations were not altered.

Results. When our modified method was compared with the method of Neuman and Logan, it was found that both methods gave the same results for the hydroxyproline content of gelatin, rat skin and rat tail tendon. Experiments in which known quantities of hydroxyproline were added to defatted rat skin and tail-tendon before acid hydrolysis yielded recoveries of 98 to 105% by both procedures. The modified procedure, however, was the more advantageous to employ since standard solutions of hydroxyproline consistently gave reproducible color intensities. This permitted the selection of aliquots from an acid-hydrolyzed tissue (in which the hydroxyproline content was approximately known) which produced color intensities lying in the more sensitive portion of the optical density scale. This was of considerable aid in the routine assay of collagenous tissue.

It should be pointed out that we have found the method not to be applicable for the determination of hydroxyproline in the presence of high concentrations of tyrosine. Neuman and Logan(3) make a correction for the tyrosine content by using its per cent color contribution (1.5% as much as hydroxyproline) after an independent determination for tyrosine. We have confirmed this value utilizing their method and have found that by our modified method, tyrosine contributes only 0.5% as much color as does hydroxyproline. However, when known mixtures of hydroxyproline and tyrosine were analyzed by either procedure, it was found that quantitative recovery of the hydroxyproline was not obtained. It was subsequently found that the light absorption of a tyrosine-hydroxyproline solution at 550 mμ was less than the summation of their individual light absorptions. Thus, a tyrosine correction will tend to give too low a value for the hydroxyproline content. In view of these considerations, it becomes important to remove high tyrosine-containing proteins from those tissues containing small amounts of collagen before hydroxyproline determinations are applied. Neuman and Logan suggest the use of a preliminary urea extraction to accomplish this purpose.

Summary. The method of Neuman and Logan for the determination of hydroxyproline has been modified by the application of ferrous sulfate to remove peroxide. This modification has yielded a consistent, linear standard curve and markedly reduced the color contribution of tyrosine.

---

1. Axelrod, A. E., and Martin, Charles J., PROC. SOC. EXP. BIOL. AND MED., in press.

2. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, 1950, v184, 299.

3. ———, *J. Biol. Chem.*, 1950, v186, 549.

4. Adams, R., and Coleman, G. H., *Org. Syntheses*, 1922, v2, 17.

Received May 12, 1953. P.S.E.B.M., 1953, v83.

Biochemical Changes in Thermally-Injured Cutaneous Tissue, Susceptibility to Proteolytic Enzymes, and Extractability of Collagen. (20387)

A. E. Axelrod and Charles J. Martin. (Introduced by Alan R. Moritz)

From the Institute of Pathology and the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, O.

A thorough knowledge of the basic biochemical changes in thermally-injured cutaneous tissue is a prerequisite to the complete understanding of the local and, perhaps, systemic reaction resulting from thermal injury. Since there exists a dearth of such information, this laboratory has undertaken to investigate these fundamental changes in the components of thermally-injured skin. It is planned to study a variety of burns, ranging from the relatively low-temperature, hot water burn to the "flash" burn which is produced by extremely short exposure to radiant energy of very high intensity. The present paper records the results of the first of these investigations dealing with the effects of a hydrothermal burn on certain properties of the protein components of skin.

Experimental. 1) *Animals.* Young adult, male albino rats of the Holtzman strain were utilized in all of the experiments described in this paper. The animals, weighing approximately 150 g when received at the laboratory, were fed the commercial dog food, "Friskies," and used within 4 months after arrival. 2) *Analytical procedures.* Total nitrogen determinations were done in duplicate by the nesslerization procedure of Miller(1) or by the micro-Kjeldahl method of Pregl. Free NH_2-N was determined by the manometric procedure of Van Slyke(2). Hydroxyproline was determined by a modification of the method of Neuman and Logan(3,4). 3. *Enzyme studies.* It has been noted frequently that the sensitivity of a protein to proteolysis reflects the degree of its denaturation. The susceptibility of skin to the proteolytic action of pepsin and trypsin was, therefore, utilized in the present studies as a criterion of the degree of protein denaturation resulting from thermal injury. Abdominal skin was utilized in all experiments. a) *In vitro thermal exposure.* The rats were anesthetized with sodium nembutal (5 mg per 100 g body weight), injected intraperitoneally, and the abdominal hair removed with an electric clipper and a razor blade. The animals were then decapitated and an area of abdominal skin (approximately 4 x 7 cm) removed and dissected free of underlying fatty and muscular tissue. This dissected skin was either used immediately or stored overnight at –30°C. In order to obtain sections of reproducible thickness, the skin was treated in the following manner. After momentary immersion in water, the skin was laid, epidermis down, on the cold stage of a specially designed sliding microtome.[†] After freezing, 15 μ sections were removed until the remaining tissue was 350 μ thick. This technic enabled us to obtain consistently a uniform sample of skin composed only of epidermal and dermal tissue as shown by histological examination. These skin sections were scissor-minced in the cold and homogenized in ice-cold distilled water, using the Potter-Elvehjem all-glass homogenizer. The resulting suspensions were sufficiently uniform and stable to permit the removal of reproducible samples. The dry matter content of these suspensions was determined by heating for 24 hours at 110°C. Suitable corrections were made for the weight of glass introduced into the homogenate by the grinding procedure. Aliquots of the skin suspensions (approximately 2 mg dry weight per ml) were heated in glass-stoppered test tubes with constant shaking in

---

[*] This project was instigated by Dr. Alan R. Moritz, and the authors wish to acknowledge his continuing interest during the course of these investigations. The work was performed under contract between the U. S. Atomic Energy Commission and Western Reserve University.

[†] This instrument was a modified motor-driven microtome equipped with a horizontal freezing stage (5 x 9 cm). With each horizontal cycle of the cold stage, it could also be advanced vertically in increments of one to 25 μ. The cold stage was cooled by a carbon dioxide expansion jet.

a water bath maintained at the desired temperature. The time required to raise the temperature of the suspension to the desired point was predetermined. Thus, all exposure times recorded in this paper represent the period during which the suspension was actually *at* the stated temperature. In all cases, the tubes were cooled in an ice bath immediately after heating. Unheated suspensions served as controls. The *proteolytic effects* of pepsin and trypsin were determined by incubating the skin suspensions for varying times with the requisite enzymes and measuring the resultant increases in total TCA-‡soluble nitrogen or TCA-soluble free NH_2-N. The concentration of skin substrate employed did not permit the maximal rate of reaction under these assay conditions. This sub-optimal concentration of skin substrate was chosen in order to avoid saturation of the enzyme, and thus permit the detection of any increased reaction rate which might result from the action of heat. The reactions were conducted in 25 ml glass-stoppered Erlenmeyer flasks shaken continuously throughout the experiment in a water bath maintained at 35.0°C ± .05°. The various substances, excepting the enzyme solution, were added as indicated in Table I, placed in the water bath and incubated for 5 minutes. The similarly equilibrated enzyme solution was then added and the incubation continued. At the desired times, 3 ml aliquots were removed and added to 4 ml of 10% TCA. Suitable autolysis and zero time controls were utilized. In the autolysis controls, the enzyme solutions were replaced with an equal volume of water or 0.003 N HCl in the pepsin and trypsin experiments, respectively. In the zero time controls, 4 ml of 10% trichloracetic acid were added prior to the enzyme to unincubated flasks containing one-fourth of the indicated amounts of each component. (Table I). After standing for one-half hour at room temperature, the trichloracetic acid suspensions were filtered through Whatman No. 3 filter paper. Anson(5) has shown that this paper does not absorb protein split-products. One or 2 ml aliquots of each filtrate were used for the determination of total nitrogen. Free NH_2-N was determined on 3 ml aliquots. In these latter experiments the concentrations of the various substances were increased proportionally in order to provide sufficient quantities of free NH_2-N for analysis. b) *In situ thermal exposures.* The abdominal hair was removed from the anesthetized rat as described in the preceding section. The following day the rat was anesthetized with nembutal and a large proportion of the abdominal area injured thermally by exposure for varying periods of time to a large volume of rapidly circulating water whose temperature was maintained within 0.01°C. A constant temperature water bath with centrally located heaters and stirrer assembly served as the heat source. An additional stirrer directly under the rat provided further mixing. The anesthetized rat was placed in a transite box with an elliptical hole which permitted the protrusion of the abdomen and a small portion of the thoracic region. The rat was sufficiently limp so that the skin-board junction was water-tight, and no other seal was necessary. Thus, the abdominal area could be burned with no injury to the rest of the body surface. The rat was placed in position and the entire assembly lowered into the water bath to a depth which maintained the water level of the bath 3-4 mm above the bottom of the box. After a stated period of time, the apparatus was removed from the bath, the animal decapitated and the uniformly burned area excised as rapidly as possible. The fri-

TABLE I. Protocol of Enzyme Experiments.

Additions	Pepsin exp., ml	Trypsin exp., ml
Skin suspension*	4	4
Enzyme†	4	4
0.44N HCl	2	—
M/10 phosphate buffer (Sorenson, pH=7.6)	—	4
Water	2	—
Final vol	12	12
Final pH	1.4	7.6
Incubation temp.	35°C	35°C

* Approximately 2 mg dry wt/ml.

† Freshly prepared solutions of Worthington's crystalline pepsin and trypsin in 0.003N HCl in concentrations of 25 and 40 μg/ml respectively. Concentration of trypsin corrected for labelled impurity of 50% magnesium sulfate.

---

‡ TCA = trichloracetic acid.

TABLE II. Effects of *In vitro* and *In situ* Thermal Exposure upon Susceptibility of Skin to Pepsin and Trypsin.

		µg trichloracetic acid-soluble nitrogen/mg dry skin						
		In vitro exposure*				*In situ* exposure†		
Enzyme	Enzyme incubation (min.)	Unheated	50°C	60°C	100°C	Unheated	55°C	70°C
Pepsin	0	10				12	10	22
		8	13	40	64	12	11	24
		12	15	36	61			
		6						
		10		32				
	10‡	35				43	41	36
		41	32	39	22	45	45	39
		36	34	30	22			
		38						
		46		33				
Trypsin	0	12				13	13	20
		9		30		18	18	24
		9		22				
		9	11		46			
		12	15	35	57			
		5		33				
	10‡	19				24	20	47
		16		29		27	28	57
		20		32				
		21	15		35			
		12	25	38	34			
		17		31				

* Suspensions held at indicated temperature one min.
† Rats exposed to bath temp. of 55°C and 70°C for 3 and 2 min., respectively.
‡ Corrected for zero time and autolysis values.

able nature of this *in situ* heated skin prevented the use of the sliding microtome for sectioning as described above. These skins, therefore, were dissected and scraped very carefully by hand in order to free them of any underlying extraneous tissue. Histological examination showed the presence of only epidermal and dermal tissue. Control animals were treated in an identical fashion with the exception that the thermal episode was omitted. The skins excised from these rats were also dissected only by hand. The preparation of the homogenates and the subsequent enzymatic procedures were identical with those described above.

4. *Extraction studies.* During the course of the enzymatic experiments, it was noted that heating a suspension of defatted, saline-extracted skin powder at 60° for one minute increased markedly the amount of nitrogen soluble in TCA. This observation led to further investigations of the effects of heat upon the ease of extraction of various components from abdominal skin and tail tendon. Loss in weight resulting from successive treatments with 0.9% sodium chloride, water, 95% ethanol, ether and 0.1 N sodium hydroxide was determined. In some cases analyses were made of the collagen contents of the extracted residues. Both *in vitro* and *in situ* thermal exposures were conducted with skin. Studies with tendon involved *in vitro* heating only. a) *In vitro thermal exposures.* Abdominal skin sections prepared as described in the *in vitro* enzymatic studies and tendon fibers of the rat tail were scissor-minced in the cold and homogenized in ice-cold distilled water. Ten ml aliquots of these suspensions (10-20 mg dry weight per ml) were centrifuged for 30 minutes in the International Centrifuge, Size 2, at 1000 g in 15 ml tared tubes, the residues resuspended in 10 ml of 0.9% sodium chloride and heated in a water bath with constant stirring. The tubes were cooled in an ice bath immediately after heating. Unheated suspensions served as controls. These saline suspensions were shaken in a water bath at 35°C for one hour and then centrifuged at 1000 g for 30 minutes. The residues were washed twice with 10 ml of distilled water, ice-cold 95% ethanol and ether at the centrifuge. The final residues

TABLE III. Effects of *In vitro* and *In situ* Thermal Exposure upon Extractability of Skin and Tendon Components.*

		% loss of original dry wt by extraction with:		
	°C	Water + saline + alcohol + ether	Same + 0.1N NaOH	% collagen on dry wt basis
		Skin		
In vitro†	Unheated	47 ± .9 (14)	66 ± .9 (14)	29 ± .8 (6)
	60	73 ± .8 (10)	89 ± 1.2 (10)	6.8 ± .1 (4)
	100	81 ± 1.2 (4)	93 ± 1.4 (4)	3.2 (2)
In situ‡	Unheated	26, 29, 29	46, 49, 49	44, 46
	50	29	49	46
	55	28	51	44
	60	41, 34, 40, 48	53, 53, 66, 75	42, 43, 33
	65	54	76	20
	70	58, 59	79, 85	16, 10
		Tendon		
In vitro†	Unheated	7.0 ± .7 (6)	34 ± .2 (4)	64 ± 1.0 (4)
	60	85 ± 1.8 (6)	95 ± 1.2 (4)	3.5 ± .2 (4)
	100	97 (2)	98 (2)	2.0 (2)

* In *in vitro* exposures, suspensions kept at stated temperatures for one min. All *in situ* exposures made for 3 min. with exception of 70° exposure which lasted for 2 min.

† Mean values ± stand. error are presented. No. of samples given in parentheses.

‡ Individual values are presented.

were dried *in vacuo* to constant weight over P_2O_5 at room temperature and weighed. The calculated percentage weight losses at this stage are given in column 2 of Table III. The residues were then extracted repeatedly with 10 ml of 0.1 N NaOH at room temperature according to the procedure of Lowry *et al.*(6). After neutralization, subsequent washings with water, alcohol and ether, and drying were conducted as described above and the residues weighed. The calculated percentage losses at this stage are given in column 3 of Table III. The collagen contents of the resulting residues were determined by suspending them in 5 ml of distilled water and autoclaving at 120 lb pressure for 8 hours(6). The residues remaining after centrifugation were washed with water, alcohol and ether, and dried in the usual manner. Since autoclaving converts collagen into soluble gelatin, the loss in weight due to autoclaving represents the collagen content of the extracted residues (Column 4 of Table III). In a few experiments, the elastin content of skin and tendon was estimated by suspending the dried, autoclaved residue in 10 ml of 0.1 N NaOH and heating in a boiling water bath for 30 minutes(6). After centrifuging, the residues were washed with water, alcohol and ether and dried as usual. This residue was considered to be elastin. b) *In situ thermal exposure.* The abdominal area was burned and skin sections prepared as described in the *in situ* enzymatic studies. Skin obtained in this manner from both burned and unburned rats was treated exactly as described in the preceding section, with the exception that the saline suspensions were not subjected to any further heating.

Results and discussion. Enzyme studies. It is apparent from Table II that the proteolytic activity of pepsin upon *unheated* suspensions of rat skin was more pronounced than that of trypsin. Since skin proteins consist mainly of collagen, these observations are in general agreement with other reports demonstrating the marked action of pepsin upon native collagen(7,8). The skin from which these suspensions were prepared contained a total of 90 μg of nitrogen per mg of dry weight. The pronounced action of heating *per se* upon the TCA-solubility of skin nitrogen is shown by the large increases in TCA-soluble nitrogen in the zero time flasks of suspensions heated *in vitro* at 60° or 100°C. In contrast to its effect on peptic activity, heating at 60° or 100°C resulted in significant increases in the proteolytic action of trypsin. This finding of an increased susceptibility of heated skin to the action of trypsin is in conformity with the observations of others that heat denatured collagen is more readily attacked by trypsin than is undenatured collagen(9,10). Experiments utilizing the changes in TCA-soluble free NH_2-N as a measure of enzymatic activity corroborated the above-described effects of heat upon the susceptibility of skin suspensions to peptic and tryptic digestion. The effects of pepsin and trypsin upon *in situ* heated skin in general paralleled those noted in the *in vitro* experiments (Table II). Thus, *in situ* exposure at 55°C produced no change in the susceptibility of the skin suspensions to peptic or tryptic digestion, whereas *in situ* exposure at 70°C resulted in a marked increase in tryptic digestion. *Extraction studies.* The increased extractability of rat skin resulting from *in vitro* exposures at 60°C and 100°C is shown in Table III. Determinations of the collagen contents of original and extracted skins by the hydroxyproline method demonstrated that the increased weight losses resulting from these thermal exposures were due to the removal of collagen. It is also apparent that the effect of heat upon the extractability of rat tail tendon was very pronounced. In the experiments cited, the percentage weight losses were computed from the weights of residues obtained by centrifugation at 1000 g. Centrifugation at 26,000 g in the Spinco centrifuge did not affect the amount of sedimentable material from tendon. In contrast, ⅓ of the skin substance rendered extractable in saline by heat was sedimented at 26,000 g. Using the hydroxyproline method on autoclaved tissue filtrates(11), it was determined that collagen constitutes 42 and 87% of unheated whole dry skin and tendon, respectively. These values are in contrast with 29 and 64% obtained by Lowry's procedure(6) which involves determination of the weight losses resulting from autoclaving the alkali-extracted residues (column 4, Table III). The lower values obtained by Lowry's procedure were found to be due to the loss in hydroxyproline-containing compounds accruing from the extraction steps preceding autoclaving. In general, the effects of *in situ* heating resembled those observed in the *in vitro* experiments. A change in extractability first became evident at 60°C and increased as the thermal episode became more severe (Table III). This increased extractability was paralleled by losses in collagen (Table III). In order to avoid the secondary complications of *in situ* heating, *e.g.*, edema formation, in some experiments the abdominal area was exposed *in situ after* the animal was sacrificed by decapitation. The effects of heat remained unaltered. The thermistor method described in the accompanying paper(12) was utilized to determine subcutaneous temperatures attained during *in situ* exposure. Subcutaneous temperatures ranging from 2-4 degrees lower than the bath temperatures were maintained for one minute or longer during the thermal episode. Elastin constituted 4% of the dry weight of skin and its quantity was unaffected by heat.

Summary. 1. The effects of *in vitro* (50-100°C) and *in situ* (55° and 70°C) hydrothermal injury upon susceptibility of the abdominal skin of rats to peptic and tryptic digestion were determined. The action of pepsin upon unheated skin was considerably greater than that of trypsin, whereas heating, both *in vitro* and *in situ*, increased only the susceptibility toward trypsin. 2. Similar hydrothermal exposures produced changes in rat skin and tail tendon collagens which rendered them extractable by normal saline.

---

1. Miller, G. L., and Miller, E. E., *Anal. Chem.*, 1948, v20, 481.
2. Van Slyke, D. D., *J. Biol. Chem.*, 1929, v83, 425.
3. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, 1950, v184, 299.
4. Martin, C. J., and Axelrod, A. E., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 461.
5. Anson, M. L., *J. Gen. Physiol.*, 1938, v22, 79.
6. Lowry, O. H., Gilligan, D. R., and Katersky, E. M., *J. Biol. Chem.*, 1941, v139, 795.
7. Sizer, I. W., *Enzymologia*, 1949, v13, 288.
8. Marriot, R. H., *J. Soc. Leather Trades Chem.*, 1926, v10, 132.
9. Haurowitz, F., Tunca, M., Schwerin, P., and Göksu, V., *J. Biol. Chem.*, 1945, v157, 621.
10. Neuman, R. E., and Tytell, A. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 409.
11. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, 1950, v186, 549.
12. Gray, R., and Axelrod, A. E., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 269.

Received May 12, 1953. P.S.E.B.M., 1953, v83.

Effect of Hyaluronidase on Experimental Hypercholesterolemia in Rabbits and Rats and Atheromatosis in Rabbits. (20388)

JOSEPH SEIFTER, DAVID H. BAEDER, WILLIAM J. BECKFIELD, GYANI P. SHARMA, AND WILLIAM E. EHRICH.

Wyeth Institute of Applied Biochemistry, and The Graduate School of Medicine, University of Pennsylvania, Philadelphia.

Both Anitchkow(1) and Aschoff(2) in their studies on atheromatosis suggested that changes in permeability of the arterial wall preceded deposition of cholesterol. Aschoff further suggested that physico-chemical alterations in the ground substance rather than simple permeability changes might be the initial lesion. Both authors agreed that the deposits were secondary. These ideas have recently been revived by Pollack(3) and Reinhardt(4) who utilized more modern methods of observing the ground substance. Since hyaluronidase affects permeability of the ground substance by altering mucopolysaccharides it was of interest to investigate the effects of the enzyme on experimental hypercholesterolemia in rabbits and rats and cholesterol-induced atheromatosis in rabbits.

Materials and methods. The regimen for producing atheromatosis was that of Bauman (5) who showed that 100% of rabbits developed the lesion after ingesting 0.43% cholesterol and 5% cottonseed oil for 14 weeks. Fifteen male and 15 female albino rabbits weighing between 1.3 and 2.4 kg were housed in individual cages and fed a basic diet of Purina Rabbit Checkers (PRC)* and tap water *ad libitum.* The animals were weighed twice weekly and their food consumption was recorded daily. For the purposes of this experiment they were divided into 5 treatment groups of 3 males and 3 females as follows:

Group A—PRC
B—PRC + 5% cottonseed oil (CSO)†
C—PRC + 5% CSO + 0.43% cholesterol (Ch)
D—PRC + 5% CSO + daily 1000 TRU hyaluronidase (H)‡ subcut.
E—PRC + 5% CSO + 0.43% Ch + daily 1000 TRU H subcut.

Total blood cholesterol was determined weekly on Manuronate®§-treated blood by the method of Bloor(6) with the following modifications. The blood was extracted twice with the 3:1 alcohol-ether mixture in glass-stoppered centrifuge tubes. The supernatant was removed after centrifuging and evaporated to the point of dryness. The residue was dissolved in redistilled reagent-grade chloroform and clarified where necessary with iron-free Norite A and recentrifuged. The clear supernatant was treated in accordance with the Lieberman-Burchard reaction. In 100 tests involving cholesterol ranges of 20-1000 mg % the results were statistically indistinguishable from those obtainable by the Sperry-Schoenheimer method(7). The *anti-hyaluronidase activity* of serum was determined by a turbidimetric assay procedure. Several 2-fold dilutions of serum were prepared in volumes of 0.4 ml in 0.1 M acetate buffer at pH 6.0. A concentration of hyaluronidase (8.4 TRU-1000 TRU/mg) that will completely reduce the turbidity of a standardized preparation of vitreous humor potassium hyaluronate was added in a volume of 0.2 ml to each tube. To each tube was also added 0.2 ml of physiological salt solution and 0.2 ml of a solution of purified hyaluronate. After 30 minutes of incubation at room temperature to allow for neutralization of the enzyme by antibodies, the residual unneutralized hyaluronidase was measured by the usual turbidimetric procedure and expressed as per cent inhibition. Normal rabbit serum and an anti-hyaluronidase serum of known potency served as controls. All sera were treated for 30 minutes at 56°C to eliminate non-specific inhibitors. The rabbits were *killed* by electro-shock and *exsanguination.* The experiment was terminated at the end of 7 weeks because the hyaluronidase treatment resulted in high antibody titers at this time. Heart, lungs, liver, kidneys, spleen, pancreas, adrenals, thyroid, pituitary, gonads, uterus, salivary glands, cervical lymph node, thymus, skeletal muscle, and marrow from the femur were removed. The organs were weighed and specimens fixed and prepared for histological examination. The aorta was dissected free to the bifurcation below the level of the adrenals and sections taken of the descending thoracic aorta and the abdominal aorta at the renal artery level. These were mounted on corks and the intima was stained with oil red 0 according to the method of Lilli(8). The sections were graded without prior knowledge of the treatment each animal had received.

---

* No cholesterol detectable by our method of analysis.

† Puritan Brand (Procter & Gamble, Inc.) contained 0.44% Ch = 0.022% Ch in diet.

‡ Wydase®, Wyeth Laboratories—7000 Turbidity Reducing Units per mg N.

§ A synthetic heparinoid anticoagulant.

The definition of grades was decided before examination of the specimens and was based on previous studies of the aortas of chickens.

0—No visible oil red 0 stain in the aorta.
1—Particles stained by oil red 0 visible with 10x hand lens.
2—Particles easily visible with naked eye after staining but few in number and discrete.
3—Large particles or plaques, no ulceration.
4—Large and ulcerated plaques.

Microscopic examination. Frozen sections stained with oil red 0 and hematoxylin from representative sections of each specimen were taken at random (preventing pathologist's view of identifying labels) and again graded on a 1 to 4 basis. Grades 1 and 2 represent small and moderate amounts of fat-stained material without displacement of the endothelium, Grade 3 larger plaques with irregular elevations of the endothelium and Grade 4 ulcerated plaques. An attempt was made to provoke the *systemic* Shwartzman reaction in 12 intact and 17 adrenalectomized rabbits. The meningococcus endotoxin which we used was kindly supplied by Dr. Gregory Shwartzman. We found that it contained no measurable cholesterol. It was administered in the doses suggested by him. Cholesterol levels were determined on blood obtained by cardiac puncture on the unanesthetized animal at appropriate intervals in the experiment as indicated in Table II. The rabbits were divided into 4 treatment groups:

Group F—6 intact rabbits received 2 intrav. inj. of meningococcus endotoxin.
G—6 intact rabbits received 5000 TRU hyaluronidase twice daily for 6 days. First dose of toxin administered intrav. on third day of hyaluronidase treatment and second dose on fourth day. All animals sacrificed 48 hr after second inj. of toxin.
H—9 adrenalectomized rabbits—same treatment as Group F.
J—8 adrenalectomized rabbits—same treatment as Group G.

The rabbits were adrenalectomized by the method of Baeder and Seifter(9) and maintained on 0.9% sodium chloride drinking water. No rabbits were used sooner than 1 week after removal of the second adrenal. The *nephrotic syndrome* was produced in 36 Long-Evans rats by injecting anti-rat kidney rabbit serum globulin free of cholesterol. The rats were divided into 4 treatment groups:

Group K—6 rats: Physiological salt sol. intraper. twice daily.
L—12 rats: 1 cc anti-kidney serum/100 g intrav. Eleven rats survived.
M—12 rats: 10000 TRU hyaluronidase daily for 4 days subcut.; on second day of hyaluronidase medication challenging dose of anti-kidney serum intrav. Eight rats survived.
N—12 rats: 100 mg streptococcal hyaluronic acid intraper. twice daily for 4 days; on second day of hyaluronate medication challenging dose of anti-kidney serum intravenously. Eight rats survived.

Seventy-two hours after administration of the anti-kidney serum to Groups L, M, and N, all the rats were anesthetized with ether, the thorax opened, and blood was obtained by

TABLE I. Data of Atheromatosis Experiment.

Sex	Group	Wt (kg) Initial	Wt (kg) Final		Total blood cholesterol levels (mg %) Wk of exp. 0	1	2	3	4	5	6	7	Degree of athermatosis in aorta Thoracic G	Thoracic M	Abdominal G	Abdominal M	Anti-hyaluronidase titer*
♂	A	2.1	3.0		97	72	51	104	92	88	107	74	0	0	1	0	—
♀		2.2	3.6		35	73	84	86	96	80	100	68	0	0	0	0	—
♀		2.0	2.9		96	94	80	99	95	86	103	71	1	0	1	0	—
♀		2.3	2.8		98	85	67	90	88	100	83	85	0	0	0	0	—
				Mean ± S.D.	82 ±28.4	81 ±30.6	70 ±17.3	95 ± 8.3	93 ± 2.7	89 ± 8.5	98 ±10.4	75 ± 7.0					
♂	B	2.1	3.1		109	35	30	92	69	64	151	87	1	0	0	0	—
♂		1.3	3.5		108	102	86	85	91	97	84	76	2	1	1	0	—
♂		1.5	3.9		34	69	105	113	84	87	99	99	1	0	1	0	—
♀		2.3	3.7		60	94	94	108	82	95	82	88	0	0	0	0	—
♀		1.6	3.1		39	108	99	92	91	77	98	91	2	0	1	0	—
♀		1.8	3.2		70	75	107	97	85	91	102	84	1	0	1	0	—
				Mean ± S.D.	70 ±36.7	81 ±28.5	87 ±29.1	98 ±10.8	84 ±10.4	85 ±12.6	103 ±25.1	88 ± 7.9					
♂	C	2.2	3.4		91	96	131	180	228	217	655	177	0	0	0	1	—
♂		2.2	3.6		103	123	156	169	197	146	526	335	1	0	1	2	—
♀		1.7	3.7		95	332	158	199	—	330	428	585	3	3	3	2	—
♀		2.1	3.6		120	357	124	147	217	282	413	533	1	2	1	2	—
♀		2.0	3.0		72	306	200	194	192	—	459	257	2	3	2	2	—
				Mean ± S.D.	96 ±19.0	303 ±13.5	155 ±26.9	178 ±20.9	209 ±21.3	244 ±25.3	496 ±98.8	377 ±175.7					
♂	D	2.2	3.2		70	112	100	90	65	64	100	120	0	0	1	1	1:80
♂		2.4	3.3		62	74	86	67	79	71	66	75	1	0	3	1	1:20
♂		2.2	3.2		71	54	104	109	64	81	98	105	1	0	2	0	1:160
♀		1.8	3.2		66	97	94	110	79	80	101	119	0	0	1	0	1:80
♀		1.5	3.3		89	88	100	91	91	82	105	95	1	1	1	1	1:160
♀		2.0	3.8		74	91	91	—	76	77	112	111	0	2	0	1	1:40
				Mean ± S.D.	72 ± 9.7	86 ±22.0	96 ± 6.8	93 ±17.9	76 ±17.8	76 ± 6.9	97 ±14.1	104 ±17.1					
♂	E	1.7	3.5		70	124	80	122	122	108	275	712	3	3	3	3	1:20
♂		2.1	3.2		113	165	74	126	109	119	302	591	2	2	2	3	1:40
♂		1.6	2.6		34	82	84	98	123	123	282	600	3	1	2	3	1:80
♀		1.9	3.8		71	309	97	145	114	125	244	660	3	3	3	2	"
♀		2.1	3.7		49	113	103	85	137	165	255	643	3	3	3	2	"
♀		1.6	2.5		92	72	138	107	121	207	244	625	3	3	3	3	1:40
				Mean ± S.D.	72 ±28.4	144 ±87.0	96 ±23.1	114 ±21.3	121 ± 9.9	141 ±12.1	267 ±22.9	641 ±12.9					

Two rabbits in Group A were sacrificed 3rd wk of exp. One rabbit in Group C died 4th wk of exp. Cause of death not determined.

* Highest dilution of serum showing at least 10% inhibition.

G = Gross grading; M = Microscopic grading.

TABLE II. Total Blood Cholesterol Levels in Rabbits Treated with 2 Intravenous Injections of Meningococcus Endotoxin.

Treatment group	Total blood cholesterol levels (mg %)				
	Control period	24 hr Hyaluronidase treatment	48 hr Hyaluronidase treatment	24 hr after 2nd toxin inj.	48 hr after 2nd toxin inj.
F	72			248	236
	84			203	209
	91			179	196
	65			198	222
	70			196	184
	81			184	192
Mean	77			201	207
G	61	74	81	135	132
	70	68	82	162	148
	69	82	71	151	129
	91	102	84	140	150
	101	96	96	178	162
	101	90	88	149	159
Mean	82	85	84	153	147
H	104			195	202
	97			199	224
	72			245	232
	84			239	201
	81			186	172
	78			201	165
	84			180	180
	71			184	175
	86			193	182
Mean	84			202	192
J	52	68	72	68	71
	101	84	86	109	98
	96	91	92	94	92
	82	84	86	88	89
	80	105	88	85	94
	85	101	100	87	88
	92	89	70	92	95
	87	84	88	94	90
Mean	84	88	85	90	90

puncture from the beating heart. The *hyaluronidase solution* was prepared fresh each day by dissolving the freeze-dried enzyme in physiological salt solution. The dose of hyaluronidase was much larger than that used clinically, and was selected to be in excess of the amount necessary to neutralize the non-specific inhibitors. The hyaluronic acid was prepared from *Streptococcus pyogenes*. It contained 3.67% N and was free from S, P, and halogen. The solutions were prepared fresh on the day of injection.

Results. The pertinent data for individual animals are found in Tables I, II, and III. For the purposes of our experiment we arbitrarily assigned a total blood cholesterol value of 150 mg % as hypercholesterolemia.

Effect of hyaluronidase and hyaluronic acid on hypercholesterolemia. Fig. 1 shows the prevention of the hypercholesterolemia due to cholesterol feeding in Group E as compared to Group C during the first 5 weeks of the experiment and the rapid rise in blood cholesterol from this time until the termination of the experiment. During the first 2 weeks the lower limit of the standard deviation of the mean total blood cholesterol levels of Group C coincided with the upper limit of the standard deviation of Group E. The standard deviation of Group C did not overlap the other 3 groups at any time during the experiment. Group E did not show definite hypercholesterolemia until the sixth week. After that it was marked and exceeded that of Group C. The abrupt rise coincided with the appearance of high anti-hyaluronidase titers (Table I).

TABLE III. Total Blood Cholesterol Levels of Nephrotic Syndrome Rats.

Treatment group	Total blood cholesterol (mg %)	Treatment group	Total blood cholesterol (mg %)
K	91	M	99
	115		96
	140		73
	112		110
	165		101
	126		90
			98
			100
Mean	125		96
L	171	N	120
	168		96
	236		104
	215		81
	248		92
	181		87
	248		90
	301		101
	241		
	331		
	301		
Mean	240		96

Hyaluronidase had a similar effect on endogenously produced hypercholesterolemia in the rabbits treated for the Shwartzman reaction (Table II) and in the nephrotic rats (Table III). In the toxin-treated rabbits the hypercholesterolemia did not depend on the presence of kidney lesions or on the adrenals. Hyaluronic acid also prevented hypercholesterolemia in the nephrotic rats. The failure to develop hypercholesterolemia did not appear to alter the course of these experimental diseases.

Effect of hyaluronidase on the degree of atheromatosis. Table I shows the results of gross and microscopic grading of aortic lipid content. The minimal change observed microscopically in aortas (Grade 1) consisted only of lipid droplets in the endothelial cells. In larger deposits (Grades 2 and 3) many large foam cells loaded with fat-stained droplets lay beneath the endothelium and elevated it. In the fat stains some material appeared to lie outside the cells but most of the lipid was intracellular. In areas with the greatest deposition, the fat-stained material was seen extending into the wall of the vessel to a depth which indicated penetration into the inner portion of the media. Groups C, D, and E showed increased deposition of lipids in the intima when compared to Groups A and B. Isolated droplets of fat-staining material also were scattered through the media in some animals in Groups C, D, and E. Foam cells were noted even in smaller deposits deep in the media.

Other than the fatty deposits it was difficult to be certain of any differences in the aortas of these animals. The general appearance was similar in the hematoxylin and eosin, Ritter-Oleson polysaccharide and trichrome stains in the several groups. Similar differences were observed in liver, spleen, and kidneys. While in liver and spleen the lipids were deposited in the Kupffer cells, in the kidney they were seen chiefly in the endothelial cells of the capillaries both in the glomeruli and in the parenchyma, but in the more severe cases many large macrophages laden with lipid were found in the connective tissue spaces between capillaries and tubules.

Discussion. The antihypercholesterolemic effect of subcutaneously injected hyaluronidase may be accounted for by 2 hypotheses. The first supposes that hyaluronidase is absorbed from the site of injection into the general circulation and is then capable of increasing the permeability of the ground substance at various sites. The greater rate of deposition of cholesterol would prevent hypercholesterolemia. The antihypercholesterolemic action of intravenously injected hyaluronidase lends support to this view. Further evidence for this hypothesis is the development

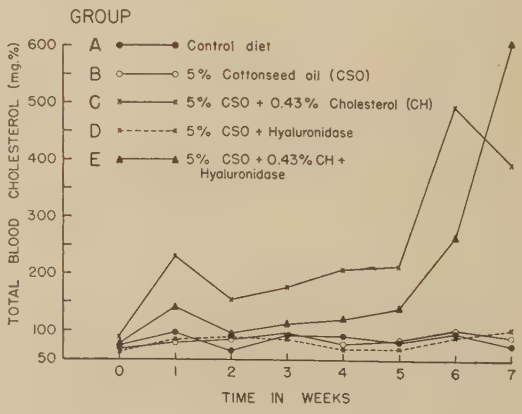


Fig. 1

of the specific anti-hyaluronidase in the circulation following prolonged administration of the enzyme. The alternative hypothesis is that hyaluronidase released hyaluronic acid from the site of injection and possibly from other sites. Hyaluronic acid has been shown to have protective colloid and dispersing action(10). It can be assumed that the hyaluronic acid released in this experiment dispersed the cholesterol in a form favoring rapid deposition. This hypothesis is supported by the fact that hyaluronic acid injected intraperitoneally suppressed the hypercholesterolemia in nephrotic rats.

The finding that the reduction of hypercholesterolemia by hyaluronidase resulted in more extensive arterial involvement casts some doubt on the treatment of human atherosclerosis by agents that lower the blood cholesterol without determining its subsequent distribution and fate. We should like to point out, however, that the morphological alterations observed in this experimental disease resemble those in human lipidoses (Xanthomatoses) rather than those of atherosclerosis. No significance can be attached at the present time to the finding of minimal intimal lesions in rabbits which received cottonseed oil and hyaluronidase without added cholesterol.

Our experiments do not reveal whether hypercholesterolemia developed following the first or second injection of endotoxin. The failure of hyaluronidase to lower the blood cholesterol level as significantly in intact rabbits as in adrenalectomized ones may be due to an anti-hyaluronate effect of the adrenal cortical hormones present in the former group.

It is of interest that a specific antibody was capable of abolishing the antihypercholesterolemic action of hyaluronidase. The circulating antibody probably neutralized the absorbed hyaluronidase before it could react at the various ground substance sites.

Facilities were not available for making ultracentrifuge examinations. If the Gofman theory(11) applies to our experiments the decrease in the blood cholesterol which is accompanied by deposition should be correlated with a decrease in the macromolecular bound cholesterol of the blood.

Summary. 1. Subcutaneous or intravenous injection of hyaluronidase depressed hypercholesterolemia in rabbits and rats. 2. Intraperitoneal injection of hyaluronic acid prevented hypercholesterolemia in nephrotic rats. 3. Hyaluronidase facilitated general lipidoses and aggravated atheromatosis in rabbits. 4. The advisability of indiscriminately lowering the blood cholesterol level in hypercholesterolemics is questioned. 5. The antihypercholesterolemic effect of hyaluronidase was abolished when specific antibodies to the enzyme developed. 6. Endogenous experimental hypercholesterolemia in rabbits does not depend on the presence of the adrenals.

We are indebted to Dr. George H. Warren for the hyaluronic acid and for determining the anti-hyaluronidase titers.

---

1. Anitchkow, N., *Deut. med. Wochenschr.*, 1913, v39, 741.

2. Aschoff, L., *Z. Ges. Neurol. Psychiat.*, 1939, v167, 214.

3. Pollack, O. J., *J. Gerontol.*, 1951, v6, 175.

4. Reinhardt, J., *Cal. Med.*, 1951, v75, 335.

5. Bauman, C. A., and Rusch, H. P., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 647.

6. Bloor, W., Pelkam, H. P., and Allan, D. M, *J. Biol. Chem.*, 1922, v52, 191.

7. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, v106, 745.

8. Lilli, R. D., *Stain Techno.*, 1944, v19, 55.

9. Baeder, D. H., and Seifter, J., *J. Pharmacol. Exp. Therap.*, 1952, v106, 372.

10. Butt, A. J., Hauser, E. W., and Seifter, J., *Cal. Med.*, 1952, v76, 123.

11. Gofman, J. W., Lindgreen, F. T., Ellent, H. A., Mantz, W., Hewitt, J., Strisomer, B., and Herring, V., *Science*, 1950, v3, 166.

Received May 13, 1953. P.S.E.B.M., 1953, v83.

Results of Exposure of Strain C3H Mice to Chloroform.* (20389)

Margaret K. Deringer, Thelma B. Dunn, and W. E. Heston.
From the National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md.

The following is a report of an accidental exposure of mice of different strains to chloroform, and of later experimental exposures of strain C3H mice to this substance with descriptions of the lesions observed. Late effects of the exposure have been determined and the experiment offers a possible explanation for some puzzling lesions of the kidney previously observed in mice(1). The most remarkable changes were found in this organ.

Accidental exposure. A large number of male mice of strains C3H, $C3H_f$ (strain C3H without the mammary tumor agent), A, and HR in some of the colonies at the National Cancer Institute died in July 1949. In one of the colonies all of the breeding males of strains C3H and $C3H_f$ died and in order to continue the strains it was necessary to use males obtained from litters of animals which had not been weaned or were born later. A smaller proportion of the strain A and strain HR males died than of strain C3H or strain $C3H_f$. No deaths occurred among the females of any of these strains nor among 57 $C3H_f$ males which had received 5-8 mg pellets of 10% stilbestrol in cholesterol several months previously. Mice of strains C57BL, C57BR/cd, C57L, and ST which were kept in the same rooms with the other strains were not affected. The kidneys of the male mice dying at that time were enlarged and pale in color, and the kidneys of those surviving longer developed calcification. The cause of these lesions was not immediately determined but it was thought possible that it was the result of accidental exposure to chloroform. A few days prior to the first death a carboy containing chloroform had been broken on the same floor where the animals were housed but at the other end of the building. Thus, any exposure must have been of low concentration although it may have lasted 24 hours or more.

Experimental exposure. Since more strain C3H males than those of any other strain had died following the accidental exposure, C3H mice were used for the experimental exposure to chloroform. The animals were exposed in an apparatus[†] which was similar in all essential aspects to that used by Werner, Mitchell, Miller, and von Oettingen(2). Following the methods used by these authors, 3 groups of 6 male and 3 groups of 6 female strain C3H mice 2 months old were exposed to a flow of air containing approximately 5 mg (ranging from 3.38 mg to 5.4 mg) of chloroform per liter of air for one, 2, and 3 hours respectively. (None of the doses administered was sufficient to produce anaesthesia in any of the animals.) In addition 3 groups of 6 male and 3 groups of 6 female strain C3H mice 8 months old were also exposed for one, 2 and 3 hours respectively to doses with the same range. Groups consisting of 22 untreated males and 20 untreated females were maintained as controls. All of the animals were housed in plastic cages and were constantly supplied with Derwood pelleted food and tap water. The animals were observed daily for deaths and were examined at weekly intervals for the presence of tumors or any abnormal condition. Autopsies were performed on all the experimental animals when they died, appeared moribund, or in respect to the females, when mammary gland tumors developed. Seventy-two experimental animals, 17 control males, and 19 control females were examined. Microscopic sections of both kidneys, the liver, spleen, lung, and mammary tumors if present were made routinely. The tissues were fixed in Fekete's modification of Tellyesniczky's fixative (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part) and stained with hematoxylin and eosin.

---

* A portion of this manuscript was prepared while one of the authors (M.K.D.) was a Summer Investigator at the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Me.

[†] The technical assistance of Wayne D. Levillain in assembling and adjusting the apparatus is gratefully acknowledged.

TABLE I. C3H Mice Exposed to Chloroform.

Conc. of chloroform, mg/l of air	Exposure, hr	Age, mo	Males		Females	
			Interval before death	Kidney lesion*	Interval before death	Kidney lesion
4.5	1	8	5 mo	L†	4 mo	0
			7	L	6	A
			7	L	8	0
4.8	1	8	5 days	E	3	0
			6	I	3	0
			8	I	5	A
5.2	1	2	15 mo	A	8	0
			18	L	10	0
			18	0	11	A
5.4	1	2	17	L	6	0
			17	L	8	0
			17	L	10	0
4.9	2	8	2 days	E	42 days	0
			6	I	49	0
			11	I	2 mo	0
4.6	2	8	1 day	E	37 days	A
			1	E	44	0
			6 days	E	7 mo	A
4.6	2	2	6	I	11	A
			6 mo	L	12	A
			14	L	13	A
4.7	2	2	2 days	E	5	0
			7	I	9	0
			7	E	11	0
5.4	3	8	2	E	49 days	0
			4	E	4 mo	0
			4	E	4	0
3.38	3	8	7	I	2	A
			7	I	3	A
			8	I	3	A
4.4	3	2	14 mo	L	8	0
			15	L	9	0
			17	L	10	0
3.84	3	2	8 days	I	8	A
			11	I	8	0
			11	I	10	0

* See text for description of early, intermediate, and late kidney lesions.

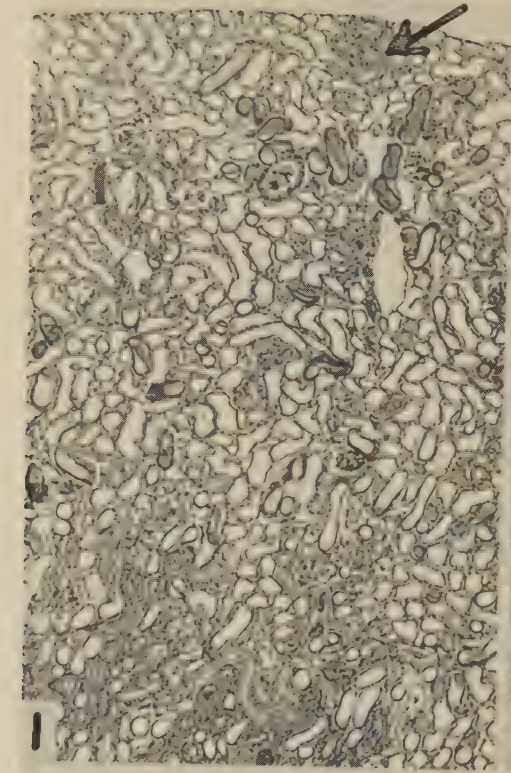
† L = Late; E = Early; I = Intermediate; A = Amyloid.

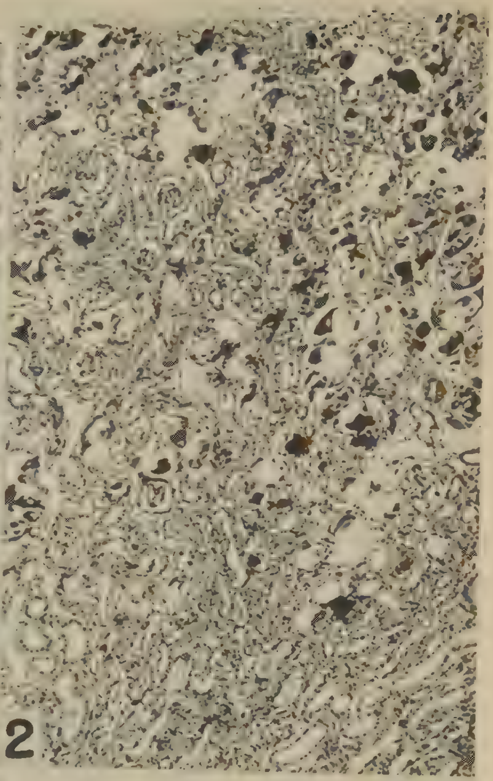
Results. The observations made after the controlled exposure to chloroform were in agreement with those made following the accidental exposure. Following exposure to air containing approximately 5 mg of chloroform per liter for one, 2, or 3 hours all of the strain C3H male mice showed gross evidence of kidney damage whereas none of the females did. Death occurred as early as one day after exposure in the 8-month-old mice and as early as 2 days after exposure in the 2-month-old group. By 11 days following exposure 15 of the 18 males of the former group and 7 of the 18 males of the latter group had come to autopsy. The remainder of the male animals exposed at 8 months of age lived from 5 to 7 months while the remainder of those exposed at 2 months of age, lived from 14 to 18 months.

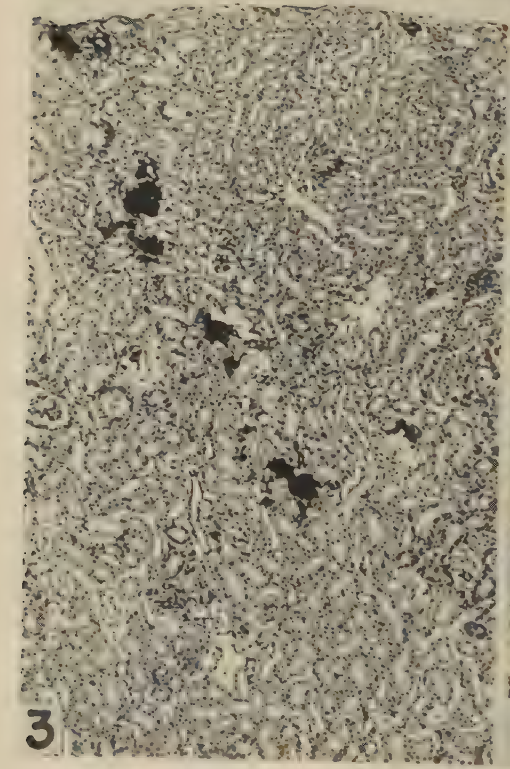
TABLE II. Strain C3H Controls.

Sex	Kidney lesion	No. mice	Avg age, mo
♂	0	6	15.5
♂	A*	11	15.4
♀	0	12	12.5
♀	A	7	13.4

* A = Amyloid.


1


2


3

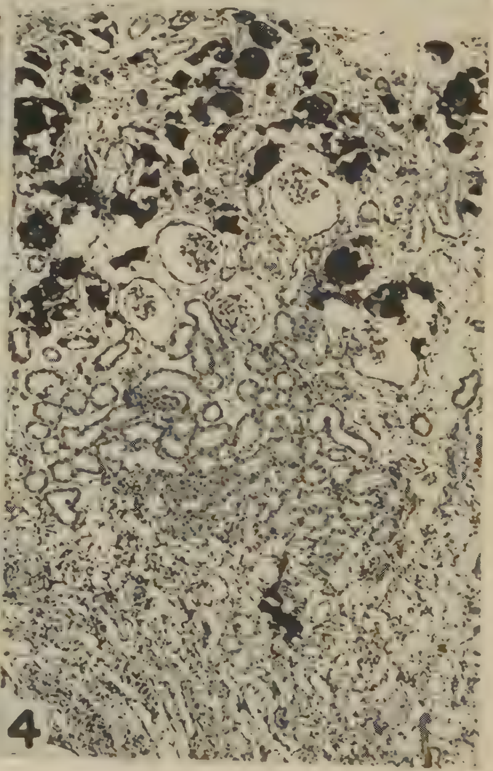

4

FIG. 1. Early. C3H ♂, death 1 day after exposure to 4.6 mg chloroform/liter of air for 1 hr. Epithelium of proximal convoluted tubules (outer zone of cortex) and of portions of distal convoluted tubules is generally necrotic. The lumens of these segments of tubules appear dilated. The glomeruli, the cuboidal lining of Bowman's capsule, and the straight portion of the proximal convoluted tubules are relatively unaffected. In a small area (marked by arrow) in the outer zone of cortex, the tubular epithelium is preserved. Occasional hyaline casts are seen. Hematoxylin-eosin. 68 ×.

FIG. 2. Intermediate. C3H ♂, death 8 days after exposure to 3 mg chloroform/liter of air for 3 hr. Necrotic areas are granular and basophilic, suggesting early calcification. Occasionally Bowman's space and tubular lumens appear dilated and the cuboidal lining cells flattened. Hyaline casts are seen in the convoluted and collecting tubules. Hematoxylin-eosin. 68 ×.

FIG. 3. Late. C3H ♂, killed 12 mo after exposure to 4.46 mg chloroform/liter of air for 3 hr. Calcified masses in cortex of a kidney which appears normal otherwise. Hematoxylin-eosin. 68 ×.

FIG. 4. Accidental exposure. HR ♂, 2 mo after exposure. Outer zone of cortex shows many calcified masses and a few surviving tubules. Many glomeruli present, Bowman's spaces greatly dilated, and normal cuboidal capsular epithelium of male is flattened. Tubules of outer zone of medulla (straight portion of proximal convoluted tubules) are dilated and the epithelium is hypertrophied. Hematoxylin-eosin. 68 ×.

An effect of the length of the time of exposure was noted in the males. Some of the animals in each group died shortly after the exposure and some lived for a considerable period of time. However, more of the males which were exposed for 2 or 3 hours died early than did those exposed for one hour. The various degrees of kidney damage were present in some of the mice after either the one-, 2-, or 3-hour exposures. Seven of the females of the group exposed at 8 months of age were killed within one to 2 months following exposure to chloroform because of the development of mammary tumors. The remainder of the experimental females lived from 3 to 13 months. Control males lived to an average age of 15.5 months and control females to an average age of 12.5 months.

Histologic observations. Descriptions of liver damage produced in mice by chloroform are already available(4). In 4 male mice dying within 1 to 6 days after exposures of 4.6 to 5.4 mg of chloroform per liter of air hepatic necrosis similar to the lesion described by Eschenbrenner(4) was found, but any such damage to the liver did not persist in males living for a longer period. No female mice died in the early period and no evidence of liver damage was seen in those dying later. No histologic changes related to chloroform exposure were found in the spleen or lungs of either males or females.

The *kidney* showed the most significant alterations. Histologic sections of the kidney were divided into the following groups: 1) Early group. Male mice dying within the first 5 days after exposure; 2) Intermediate group. Male mice dying from 6 to 11 days after exposure; 3) Late group. Male and female mice surviving for a period of several months; 4) Amyloid group. Unexposed control and exposed male and female mice which showed little or no calcification of the kidney, but had an extreme degree of amyloid infiltration; 5) Normal group. Unexposed control and exposed male and female mice in which the kidneys appeared normal; and 6) Accidental exposure group. Male mice which had been accidentally exposed and survived for a number of months. A characteristic picture for each group can be described. In Table I the experimental animals of the various exposures are classified according to the type of kidney lesion found. The controls are classified in Table II.

Changes in the *early group* where death occurred within 5 days are shown in Fig. 1, and characteristic features are pointed out in the legend. Although not shown in the figure, a short segment of the tubule leading from the glomerulus was also preserved as were the nuclei of the cells of the macula densa although the remaining portion of the tubule was sometimes necrotic. The epithelium of the descending straight limb of the proximal convoluted tubule was generally spared, as was that of Henle's loop and of the collecting tubules. In the *intermediate group,* in which death occurred in from 6 to 11 days, the kidneys showed less extensive damage (Fig. 2). In addition to features noted in the legend mitoses were very infrequent. Characteristic features of the kidneys of mice in the *late group* that died several months after exposure are shown (Fig. 3). The mice which were in the controlled exposure group showed less extensive alteration than did many of the accidentally exposed mice which lived for a time after exposure (Fig. 4).

Amyloidosis was found in both control and exposed mice in which death occurred late. The kidney lesion with amyloidosis has been described previously(1). The tip of the papilla was often necrotic, and there was amyloid deposition in the glomerular tuft. In some kidneys of the male animals exposed to chloroform both the typical amyloid lesion and scattered patches of calcification were found. The kidneys were *normal* in 12 of the 19 control females, in 6 of the 17 control male mice, and in 24 of the 36 exposed females. No calcification was found in any of the kidneys of the females but amyloidosis was found in 12 instances. Mice of resistant strains (C57BL, C57BR/cd, C57L, and ST) and $C3H_f$ males with stilbestrol pellets all of which survived the accidental exposure and which were autopsied at a later date showed no renal calcification.

Discussion. Susceptibility to exposure to chloroform increased with increase in age of the mice for the strain C3H males exposed to various doses at 8 months of age showed greater response than did those exposed at 2 months of age. Furthermore, young male mice which were not yet weaned at the time of the accidental exposure were not affected. Length of exposure had an effect in that a larger number of animals died within a few days following the two and three hour exposures than following the one hour exposure.

The differences between the responses of the various inbred strains to the accidental exposure to chloroform indicated a genetic difference in response. The susceptible strain A is distantly related to strain C3H, and thus $C3H_f$, in that A and C3H were derived from 2 outcrosses of Bagg's colony of albinos(5). The resistant strains, C57BL, C57BR/cd, and C57L are also related. Strains C57BL and C57BR were developed by inbreeding the black and brown segregants of a cross between mice of a heterogeneous stock and strain C57L arose as a mutation in the C57BR line. As far as is known, neither the susceptible strain HR nor the resistant strain ST are related to any of the other strains mentioned.

The difference in the response of the 2 sexes to an exposure to chloroform in this experiment and in the accidental exposure may be compared with the findings of Eschenbrenner (4), and Eschenbrenner and Miller(3). They observed that in mice receiving oral administration of chloroform necrosis of the convoluted tubules of the kidneys was present in intact males and testosterone-treated castrated males but not in females. Eschenbrenner(4) not only noted the difference in response of male and female mice to chloroform, but also briefly described the early kidney lesion which affected "portions of both proximal and distal convoluted tubules". He noted that the cuboidal cells lining Bowman's capsule were never necrotic.

A similar effect of chloroform has recently been reported by Shubik and Ritchie(6) who observed that male DBA mice housed in a room where animals were sacrificed by chloroforming them, died within 6 days. Large, pale kidneys were noted at autopsy. On microscopic examination, necrosis of the tubules of the kidneys was seen. Females of strain DBA and mice of both sexes of strains A, BALB/c, C3H, and CAF_1 hybrids housed in the same room, were not affected by the exposure to chloroform.

A point of interest in the histopathologic study of the kidney lesions in the present study was the resemblance which was found to previously observed "idiopathic calcification" in the kidneys of mice(1). In the idiopathic lesion a remarkable sex difference was noted; calcification in the kidneys of the males involved the whole outer zone of the cortex, whereas in those of the females it was restricted to a few flecks of calcium at the cortico-medullary junction. In the experimental exposure to chloroform, the kidneys of the females showed no calcification, but in those of the males the calcification was similar to that in the idiopathic type.

The localization of damage to particular segments of the tubule is similar to that described after poisoning with bichloride of mercury, uranyl nitrate, and potassium dichromate(7). The glomeruli after chloroform exposure appeared to be resistant, and the cells of the macula densa were preserved, even when the rest of the cells lining the tubule at the same level were necrotic. In the animals which survived, it seems probable that a sufficient portion of the kidney remained unaffected to maintain life, since very little evidence of regeneration was found. The fate of the glomeruli after destruction of the tubules is uncertain but many appeared to survive, even though all tubules in an area had undergone necrosis or calcification.

No exact relationship was found between the areas of initial necrosis with later calcification, and the localization of alkaline phosphatase in the tubular epithelium which has been studied in sections from kidneys of normal mice(1). The cuboidal epithelium lining Bowman's space, for example, is heavily blackened when Gomori's technic is used to determine alkaline phosphatase yet almost no necrosis and calcification occurred at this site. This observation supports the studies of Hepler and Simonds(7) in dogs in which phosphatase did not appear to be concerned with calcification in the kidneys.

The alteration produced by chloroform should be useful in studies of kidney damage and repair, as suggested by Eschenbrenner and Miller(3). The sex difference offers an especial opportunity to study further the effect of hormonal alterations upon the injury to the kidney produced by chloroform as observed in the accidentally exposed $C3H_f$ males bearing stilbestrol pellets.

Summary. 1. Exposure of strain C3H mice to air containing approximately 5 mg of chloroform per liter for one, 2, or 3 hours resulted in lesions of the kidneys of all of the males but in none of the females. Histologic details of this lesion were described. 2. The lesion found in the male animals in this experiment was similar to that exhibited by the males of strains C3H, $C3H_f$, A, and HR, some of which died immediately after an accidental exposure to chloroform and some in the months following the exposure. 3. A genetic difference was apparent in that whereas strains C3H, $C3H_f$, A, and HR were susceptible, strains C57BL, C57BR/cd, C57L, and ST were resistant to the accidental exposure.

1. Dunn, T. B., *J. Nat. Cancer Inst.*, 1949, v9, 285.
2. Werner, H. W., Mitchell, J. L., Miller, J. W., and von Oettingen, W. F., *J. Ind. Hyg. and Toxicol.*, 1943, v25, 157.
3. Eschenbrenner, A. B., and Miller, E., *Science*, 1945, v102, 302.
4. Eschenbrenner, A. B., *J. Nat. Cancer Inst.*, 1945, v5, 251.
5. Heston, W. E., *Twentieth Commemoration Lectures on Genetics, Cancer, Growth, and Social Behavior*, Roscoe B. Jackson Memorial Laboratory, 1949, 9.
6. Shubik, P., and Ritchie, A. C., *Science*, 1953, v117, 285.
7. Hepler, O. E., and Simonds, J. P., *Arch. Path.*, 1945, v40, 37.

Received May 15, 1953. P.S.E.B.M., 1953, v83.

Screening of Chicks with Erythromyeloblastic Leukosis for Plasma Activity in Dephosphorylation of Adenosine Triphosphate.* (20390)

E. B. Mommaerts, Dorothy Beard and J. W. Beard.

From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

Dephosphorylation of adenosine triphosphate by the blood plasma of chickens diseased with erythromyeloblastic leukosis has been described(1). In qualitative experiments, it

---

* This work was supported by a research grant to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by a grant from the American Cancer Society, on recommendation of the Committee on Growth; by the Dorothy Beard Research Fund; and sponsored by the Commission on Influenza, Armed Forces Epidemiological Board, and supported, in part, by the Office of The Surgeon General, Department of the Army.

appeared likely that this activity was related to the presence of a particulate component (2,3) which has been judged to be identical with the etiological virus of the condition. This possibility has been greatly strengthened by the results of quantitative experiments currently in progress which show, unequivocally, an intimate relationship between enzymatic activity, particle number and the infectivity of the plasmas from diseased chicks.

The occurrence of the enzyme suggested the development of a rapid micro test for the screening of diseased birds for individual plasmas of high activity. A simple procedure, designed and employed routinely for this purpose, is described in this report.

Materials and methods. The origin of the virus and the characters of the disease under study have been described(4,5). All of the present results were obtained with chicks of a special strain of White Leghorns (strain 15 (6)) obtained from the U. S. Regional Poultry Research Laboratory, East Lansing, Mich. The chicks tested were from various routine titration experiments in which they had received filtered plasma from previous passage birds given intravenously at 3 days of age. *Selection of the chicks* for the test was made on the basis of the number of circulating primitive cells as judged by examination of blood smears(4). Blood was obtained by needle prick of the wing vein and drawn into capillary tubes 0.8 to 1.1 mm I.D. and 68 mm long (Kimble Glass No. 46486). Clotting was prevented by the film of heparin ("liquaemin", Roche-Organon, 1 ml = 5 mg) left in the tube by drawing in the liquid and expelling it immediately before the sample was taken. The blood was allowed to flow away from the end of the tube which was then sealed in a flame. The cells were sedimented in a refrigerated centrifuge at 1029 x g for 15 minutes. This was done with special holders, each made from an aluminum cylinder 53 mm long and 17 mm diameter and fitting a standard centrifuge cup. A set of 15 holes 38 mm deep was bored in one end to take the capillary tubes, a space being left as index point, and a handle was fitted to the cylinder for insertion and removal from the centrifuge cup. When the *cells had been sedimented,* measurements of the relative plasma, primitive cell and red cell volumes were made with a mm scale which provided an approximate estimate of the hematocrit value. The glass was scratched about 5 mm above the white cell layer, the tube was broken and a sample of 5 mm^3 (λ) was taken with a fine pointed pipet(7). This was carefully deposited at the bottom of a glass tube 100 mm long and 6 mm I.D. The tubes must be entirely free of acid or base. An *indicator solution* was prepared by dissolving in distilled water the disodium salt of adenosine triphosphate (Pabst Laboratories, Milwaukee, Wis.) in a concentration of 3 mg per ml. The pH was adjusted to 8.2 with 0.1 N KOH. For each ml of this solution, 0.2 ml of brom thymol blue (0.04%, Hartman-Leddon Co., Philadelphia) was added. It was convenient to prepare the solution in 25 ml lots and to store aliquots of 3 ml frozen. The solution could be kept in this manner for several months. Thawing and refreezing of the contents of a tube from day to day could be repeated about 3 times without destroying the usefulness of the preparation. To each tube containing 5 λ of plasma, 0.12 ml of the indicator solution was added; the mixture was frequently shaken during the reaction, and the time was recorded for the reaction to progress to the point where the color of the test mixture matched that of a control preparation of brom thymol blue of pH 6.6.

It was desirable to learn the accuracy of these results by comparison with those obtained by titrimetric measurement of the acid liberated during the enzymatic dephosphorylation. The method employed was that of Green and Mommaerts(8). For the titrimetric determinations, blood was taken in chilled, pointed centrifuge tubes by heart puncture or section through the sagittal sinus. Clotting was prevented by the presence of heparin, 1.0 mg per 10 ml blood, and separation of the cells from the plasma was accomplished by centrifugation first at 1029 x g for 15 minutes and then by spinning the resulting plasma in celluloid tubes at 3715 x g for 15 minutes. Great care was taken in pipetting the plasma after the second centrifugation to avoid the few residual cells. Plasma samples of 0.05 to 0.2 ml were used for the determination. A volume of 0.02 ml of 1.0 M $CaCl_2$ was added to a volume of Ca-free Ringer solution calculated to make, together with the amount of plasma used, a total volume of 19.0 ml. To this the plasma was added, and the mixture was incubated 3 minutes at 30°C after which 1.0 ml of adenosine triphosphate was poured in. The adenosine triphosphate solution, at pH 9, contained 4 mg of the disodium salt per ml. From this point, following the procedure of Green and Mommaerts (8), the rate of the reaction was observed by pH measurement, with glass electrodes and Beckman pH meter, during successive additions of 0.05 ml portions of 0.01 N KOH to a total volume of 0.3 ml, using pH 8.0 as the index point. Individual points in the rate line were expressed in terms of the time needed for the neutralization of each aliquot of base. By appropriate calculation, these data were transformed to an expression of the rate of liberation of phosphorus by unit volume of plasma. In a few experiments, the results obtained by this procedure were checked and proved to be identical with those employing the direct determination of phosphorus with the method of Berenblum and Chain(9). A 5 λ sample of the same plasma was taken for the final estimation with the micro test.

Results. The micro screening test described above has been employed routinely for the past 4 months, and the results obtained with the plasmas from 1128 chicks have been tabulated for the present report. Under the conditions indicated, the time required for the reaction to reach the end point has varied from 1.5 minutes, in rare instances, to periods of 15 minutes and longer. In many cases there was little or no evidence of any reaction. Observation was terminated at the 15-minute point, since, except for special purposes, plasmas of activity lower than that needed to complete the reaction within this period were of no value in the experiments for which the screening was done.

The distribution of reaction times of the plasmas under study is shown in Table I. With approximately 50% of the plasmas, the end point of the reaction was not reached within the arbitrarily chosen period of 15 minutes. The percentages of plasmas giving the end point within the 15-minute period were essentially the same for the 5-, 10-, and 15-minute times.

TABLE I. Time Required for Individual Plasmas from Chicks with Erythromyeloblastic Leukosis to Lower the pH to the End Point of 6.6 in the Micro Test.

		Time of reaction—min.			
	Total	<5	<10	<15	>15
No. plasmas	1128	176	215	184	553
%	100	15.60	19.06	16.31	49.03

A comparison of the results of the micro test with those in the accurate determination of the enzymatic activity of the plasmas in dephosphorylating adenosine triphosphate is shown graphically in Fig. 1. These 50 plasmas were selected by preliminary micro test to afford a good distribution over the range of the most active specimens to others in the lowest range accessible to study with the volume, 5 λ, employed. In making the observations indicated in Fig. 1, the micro test was repeated on the centrifuged plasma used for titration measurement. It is evident that the relation of the reciprocal of the reaction time estimated in the micro test is linear with the phosphorus liberated as measured by titration. Variation in the results of the micro test, with respect to the titration estimations, seems uniform throughout the range. The area enclosed within the discontinuous lines represents a fiduciary band indicating the limits of the expected error of a single determination by the micro method. Thus, in such a single determination there would be expected in 2 of 3 cases an error of ±75 gamma phosphorus when the conditions of measurement are maintained as described. Since the absolute error is uniform, the percentage error becomes progressively smaller in the range of higher plasma activities.

The relation of the primitive cell content of the blood to the enzymatic activity of the plasma observed in the micro test is shown in Table II. It should be emphasized that the measurements were only approximate. The line of separation between the red and the white cells was never clear cut, and in many instances the relative volume of red cells could only be a matter of guess. It is likely, nevertheless, that the data of Table II are adequate

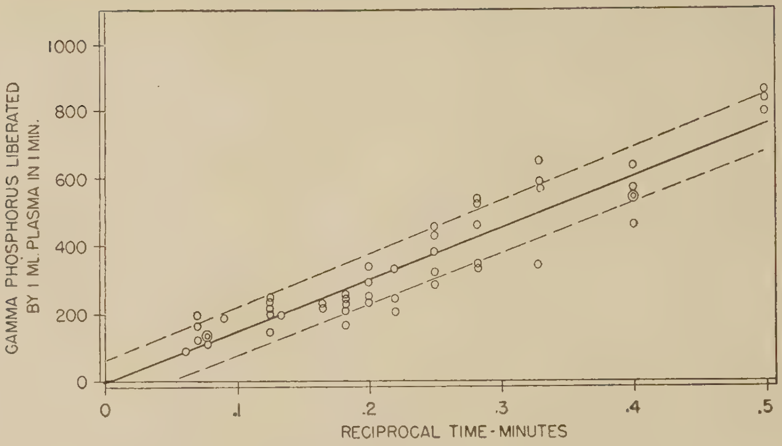


FIG. 1. Relation of liberated phosphorus measured by titrimetric determination to reaction time found in the micro test.

in revealing the essence of the relation. In the results with 609 specimens of blood, the activity of the plasma is related statistically to the concentration of primitive cells. However, it is likewise evident that the variation in the activity of the individual plasmas about the mean is enormous and overlapping. The individual variations cited in Table II indicate the limits to be expected of the results of 2 plasmas of 3 tested. The most that can be said about the relation is that while high activity is never present in the plasma of a blood of low primitive cell count, a high cell concentration is not a criterion of a high activity.

Discussion. The usefulness and ultimate application of the micro test described here, or improved modifications of it, are dependent on a number of factors. In routine work with the virus of erythromyeloblastic leukosis which is dependent on access to plasmas of high particle content and infectivity, the procedure is of much value. The particle content of the plasmas varies from about 10^9 per ml in "15-minute" plasmas to more than 10^{12} in those of 1.5 to 2-minute reaction times. This has been clearly established in an adequate series of experiments to be reported elsewhere. It can be said also that the results of the micro test provide an index of the range of infectivity of the plasma. The variations in the degree of correlation between dephosphorylation of adenosine triphosphate and infectivity are not known as well as those between particle content and enzyme activity since the number of titration experiments has been limited because of practical reasons. Nevertheless, in every instance thus far tested, high infectivity has been associated with high enzymatic activity. When it is desirable to select plasmas of high particle count and infectivity, the micro test makes possible the rapid elimination of approximately 80 to 90% of the specimens which might be regarded as useful by any other criterion. In principle, the test is applicable to the study of erythromyeloblastic leukosis in the same way as the hemagglutinative reaction is to the investigation of the influenza virus. The test possesses the advantages of much simpler application for semi-quantitative data which are probably more accurate than those obtained with hemagglutination by the

TABLE II. Approximate Hematocrit Values, in %, of Blood Samples in Relation to Time Periods Required for the Reaction to Reach the End Point in the Micro Test.

	Time of reaction—min.			
	<5	<10	<15	>15
No. chicks	107	108	93	301
RBC, Mean	11.3	11.5	13.8	16.1
WBC, "	46.5	42.5	36	23.5
S.D. of mean	±1.09	±0.97	±1.41	±1.56
Individual variation*	35–57	32–52	22–50	0–49
Plasma	42.2	46	50.2	60.4

* These values represent the limits in variation of results to be expected with 2 of 3 plasmas tested.

influenza virus. This cannot be judged finally since comparable data are not available for the hemagglutinative reaction. The fact that the absolute relationship of the enzymatic activity to the particle number and to infectivity is not fully understood does not detract from the usefulness of the test.

Use of the smallest possible volumes for the test is necessary since the chicks yielded on bleeding about 1.5 to 2 ml of plasma and even a minute drain on the circulating blood frequently leads to death of the chick before the test can be completed. It happens that with the capillary tubes employed, the volume of plasma from the best chickens was only 5 λ or 10 λ. The range of activity measurable could be extended by the use of 10 λ volumes, but in practice this has not seemed desirable.

Summary. A micro procedure has been designed for the rapid testing, in 5 λ volumes, of the plasmas of chickens with erythromyeloblastic leukosis for their capacity to dephosphorylate adenosine triphosphate. Since this enzymatic activity is related both to the number of virus particles and to the infectivity of the plasmas, the test provides the means either for the measurement of those qualities or for the selection of chicks possessing the qualities in the desired degree. There is a wide variation in this enzymatic activity among the individuals of the diseased chick population. For practical application to the study of erythromyeloblastic leukosis, the test is comparable with the hemagglutinative reaction with the influenza virus. The test in its present form would not be useful in the diagnosis of erythromyeloblastic leukosis which can be accomplished much more easily by blood smear. No experiments have been made to learn whether the reaction might occur with the plasma of birds with visceral lymphomatosis which is much more difficult to diagnose in the intact bird.

---

1. Mommaerts, E. B., Eckert, E. A., Beard, D., Sharp, D. G., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 450.
2. Beard, D., Eckert, E. A., Csaky, T. Z., Sharp, D. G., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 533.
3. Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W., *J. Bact.*, 1952, v63, 151.
4. Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Can. Inst.*, 1951, v12, 447.
5. ———, with an appendix by Burmester, B. R., *J. Nat. Can. Inst.*, 1953, v13, 1167.
6. Waters, N. F., *Poultry Science*, 1951, v30, 531.
7. Eckert, E. A., Reeve, R. H., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 113.
8. Green, I., and Mommaerts, W. F. H. M., *J. Biol. Chem.*, 1953, v202, 541.
9. Berenblum, I., and Chain, E., *Biochem. J.*, 1938, v32, 925.

Received May 20, 1953. P.S.E.B.M., 1953, v83.

Tubeless Gastric Analysis by Use of Insoluble Salts with Exogenous Ions.[*]
(20391)

HARRY L. SEGAL, AND LEON L. MILLER. (Introduced by William S. McCann)

From the Departments of Medicine and Biochemistry, the Strong Memorial, Rochester Municipal and Genesee Hospitals, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Tubeless gastric analysis was introduced by Segal, Miller and Morton(1). The principle was based upon the appearance in the blood, urine and/or saliva of a non-toxic exogenous indicator ion after its release from an insoluble indicator compound by the action of the hydrogen cations of gastric juice. It was also suggested that the special indicator ion might be a dye, a radioactive material or any easily detectable substance. Extensive experience with the oral administration of an insoluble indicator compound[†] comprised of a cation exchange resin with the quininium cation as the specific exogenous indicator ion, has proved the efficacy of this technic(2-6). *In*

---

[*] Supported by a Special Medical Research Fund.

[†] Marketed as Diagnex by E. R. Squibb & Sons, New York.

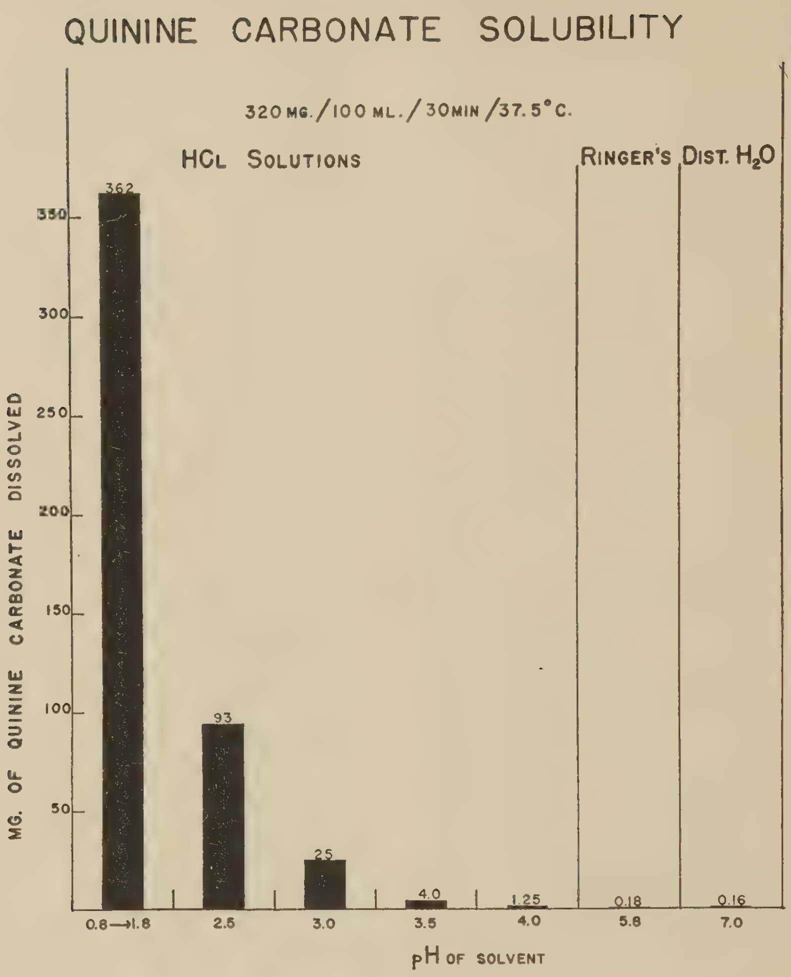


FIG. 1.

vitro and preliminary clinical investigations of other insoluble indicator compounds prepared with ion exchange resins and various special exogenous indicator ions as methylene blue, serenium, pyridium, fluorescein have also been reported(7). Similarly, tubeless determination of gastric acidity by the oral administration of insoluble salts such as calcium carbonate and magnesium pyrophosphate containing Ca 45 and P 32, respectively, as labeled endogenous indicator ions has been described by Maurer and Zimmer(8). Obviously, the detection of tagged endogenous ions in such compounds is not generally applicable.

The present study is concerned with the use of quinine carbonate as an example of an insoluble salt with a special exogenous indicator ion which by its solubility in hydrochloric acid permits the detection of free gastric acid without subjecting the individual to intubation. *Quinine carbonate* $CO_3(C_{20}H_{23}O_2N_2)_2$ is an amorphous white substance with a molecular weight of 674.39 and containing 96.14% of quinine. It is practically insoluble in water and Ringer's solution and soluble in hydrochloric acid. One milliequivalent of the available quininium cations of this compound should react with 1 mEq of the hydrogen cation of hydrochloric acid to form 1 mEq of quinine hydrochloride. Studies in this laboratory have shown that from 0.3% to 1.4% (average 1%) of the quinine of quinine hydrochloride administered orally will be excreted in the first 2 hours' urine and from 0.8% to 2.2% (average 1.6%) in the first 3 hours' urine. Hence, if the quinine carbonate administered orally were available to react with all of the gastric hydrochloric acid secreted per unit of time, a quantitative assay of the quinine excreted in the urine 2 or 3 hours after the oral administration of quinine carbonate should afford an approximation of the hydrochloric acid secretion per unit of time. This is at best an approximation since the orally administered quinine carbonate and the gastric juice may both pass into the duodenum before complete stoichiometric reaction can occur, and since the excretion range of quinine varies from 0.3 to 1.4% in the first 2 hours' urine. Clinically, this should suffice to differentiate between degrees of acidity and achlorhydria, if sufficient quinine carbonate can be administered orally.

Methods. A) *In vitro* experiments. The following technic was used to test the solubility of quinine carbonate in hydrochloric acid of varying hydrogen ion concentration, in Ringer's solution and in water. To 32 mg of quinine carbonate in a test tube were added 10 ml of the solution to be examined. Temperature was kept constant at 37.5°C and the mixture was stirred with a slow stream of air for 30 minutes. The solution was then filtered through Whatman No. 5 filter paper and 5 ml of the filtrate were placed in a 60 ml capacity separatory funnel. 1 N NaOH was added until the pH was greater than 11. Fifteen milliliters of ether were then added and mixed gently. Enough ether was then withdrawn to allow 8.2 ml to remain in the funnel. Five milliliters of 0.1 N H_2SO_4 were next mixed with the 8.2 ml of ether. The acid solution which now contains the quinine was drawn off and diluted with the appropriate amount of 0.0015 N H_2SO_4 to read on the Lumetron photofluorometer (standard 2 μg). The percentage of error is ±10%.

Results. Fig. 1 reveals results of the test of the *in vitro* solubility of quinine carbonate multiplied by 10 to show the extent of solubility of 320 mg of quinine carbonate in 100 ml of the solution tested.

B) *In vivo* experiments. *In vivo* experiments were performed by administering orally different doses of quinine carbonate mixed in about 60 ml of water. Five hundred milligrams of caffeine sodium benzoate were used as the gastric stimulant for individuals in whom the gastric secretory response to caffeine had been determined previously, either by the usual intubation technic or by the quininium indicator compound tubeless method(2-6) on a day other than that of the quinine carbonate test.

The *first group*, consisting of 24 individuals who secreted at least a normal amount of free hydrochloric acid per hour after caffeine stimulation and seven achlorhydric patients, were given a dose of 120 mg of quinine carbonate. Urine was collected before and 2 hours after oral administration of the compound. The quinine was extracted from the urine by a simplified modification(4,7) of the Kelsey-Geiling ether-sulphuric acid technic(9) and determined quantitatively with the Lumetron photofluorometer as described in the *in vitro* tests. For clinical purposes, micrograms of quinine less than 160 can be estimated semiquantitatively by comparing the fluorescence with appropriate standards using a Blak-Ray or other type of lamp as the ultraviolet source (4,7). To a *second group* consisting of 33 individuals who secreted free hydrochloric acid to at least a normal extent, 3 who secreted hydrochloric acid in the low range, 10 achlorhydrics and 2 individuals with gastrectomies, 320 mg of quinine carbonate was administered orally. A *third group* made up of 7 acid secretors, one low acid secretor, 3 achlorhydrics and 2 gastrectomized individuals received 640 mg of quinine carbonate.

With one exception the 320 and 640 mg groups had urine collected before, 2 hours, 3 hours and 5 hours after the oral administration of the quinine carbonate. In one hyperacid secretor, one low acid secretor and one achlorhydric (P.A.) patient, additional urines were collected at 8, 15 and 24 hours after the oral administration of 320 mg of quinine carbonate. In one gastrectomy patient, urine was collected 2 hours and 10 hours after the oral administration of 640 mg of quinine carbonate.

In the acid secretors of the group receiving 120 mg of quinine carbonate, the average amount of quinine in the first 2 hours' urine was 72 μg with the range from 0 to 192 μg.

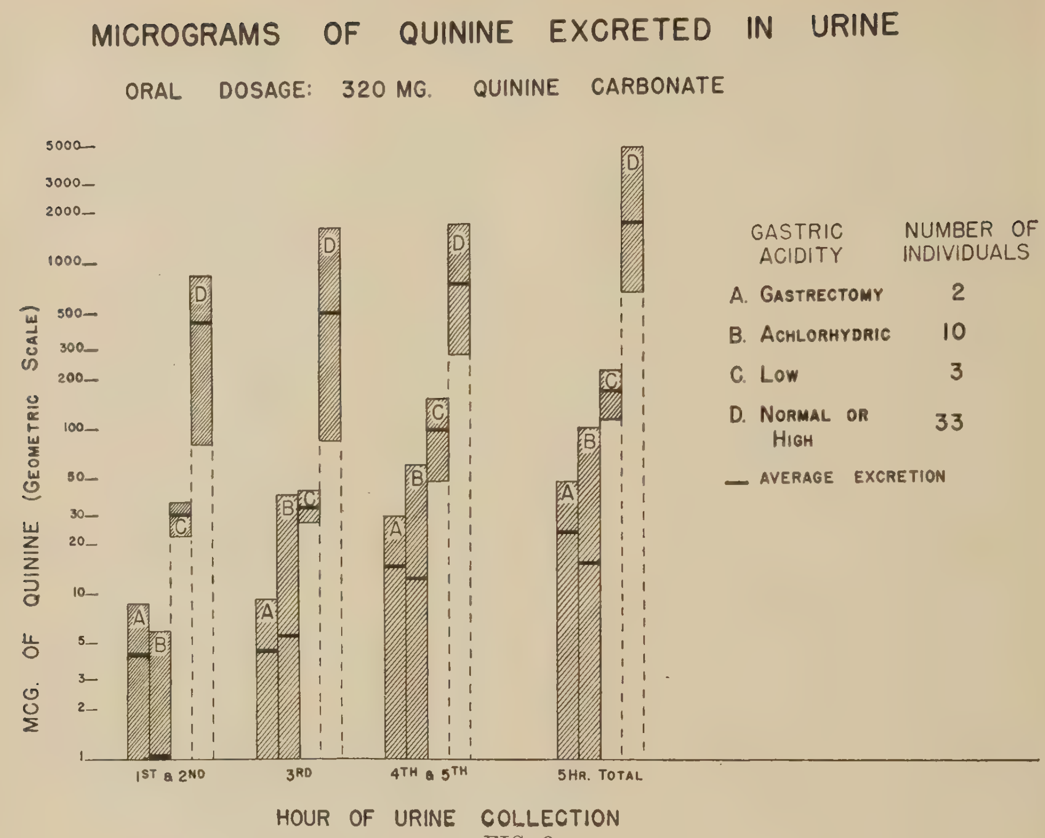


FIG. 2.

All 7 of the individuals with achlorhydria failed to excrete any quinine.

Fig. 2 demonstrates the results in the group receiving the 320 mg dose. The acid secretors in the normal or higher range excreted an average of 437 μg of quinine in the first 2 hours' urine as compared to 72 μg excreted in the comparable group receiving 120 mg. The amount of quinine in the 2 hours' urine varied from 80 to 840 μg. The average quinine excreted in the first 2 hours' urine was 30 μg in the low acid secretors and 1.1 μg and 4.3 μg in the comparable urines in the achlorhydric and gastrectomy patients, respectively. Slightly larger amounts of quinine were excreted in the third hour and in the fourth plus fifth hour periods.

In one P.A. patient the amounts of quinine in the 8th and 15th hour urines after 320 mg of quinine carbonate were zero in both collections and 114 μg in the total 24-hour urine. In the one low acid secretor it was 36 μg, 27 μg and 285 μg in comparable urines; in the one high acid secretor, 999 μg, 780 μg and 3,726 μg, respectively, were found in the urines.

The average μg of quinine excreted in the group which received 640 mg of quinine carbonate were as follows:

	Time of urine collection		
Gastric acidity	2nd hr	3rd hr	5th hr
Normal or high	1030	1070	2170
Low	54	156	120
Achlorhydric	0.6	2	23
Gastrectomy	0	0	6

In one gastrectomy patient who received 640 mg of quinine carbonate, no quinine was excreted in the first and second hours' urine and only 23 μg were found in the total 10-hour urine.

The data obtained show that the determination of the amount of quinine in the first 2 hours' urine excretion after the oral administration of 320 mg of quinine carbonate can serve to detect the presence or absence of free gastric hydrochloric acid without subjecting the individual to intubation. Under proper conditions with this dose of quinine carbonate the differentiation between a low and at least a normal acid secretor seems feasible merely by assaying the 2-hour urine for quinine by the simple semi-quantitative technic previously described(4,7).

Experimentation with larger doses of quinine carbonate, if tolerated, or with other comparable insoluble salts with exogenous indicator ions, may make it possible to differentiate approximately between normal and excessive output of hydrochloric acid per unit of time. It is obvious that the time of urine collection can be changed to include the night excretion if desirable, since in achlorhydric and gastrectomized patients practically no difference was noted in the amount of quinine excreted in the 2-, 5- and 8-hour urine collections.

Summary. 1. The rationale of the use of insoluble salts with special exogenous indicator ions to determine gastric acidity without intubation has been described. 2. Theoretical consideration and *in vitro* and *in vivo* experiments have demonstrated that by determining the amount of quinine in the first 2-hour urine excretion after the oral administration of 320 mg of quinine carbonate, the presence of free hydrochloric acid from a low to a normal or higher range or its absence can be estimated without subjecting the individual to intubation. 3. Further experimentation with larger doses of quinine carbonate, if tolerated, or with comparable insoluble salts with various exogenous indicator ions, may make it possible to differentiate approximately between normal and high gastric hydrochloric acid secretion without intubation.

The authors wish to express their appreciation for the technical aid given by Miss Elizabeth Plumb and Mrs. Elizabeth Arrowsmith.

---

1. Segal, H. L., Miller, L. L., and Morton, J. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 218.

2. Segal, H. L., Miller, L. L., Morton, J. J., and Young, H. Y., *Gastroenterology,* 1950, v16, 380.

3. Segal, H. L., *Med. Clinics of N. A.,* 1951, v35, 593.

4. Segal, H. L., Miller, L. L., and Morton, J. J., *J. Nat. Cancer Inst.,* 1953, v13, 1079.

5. Malach, M., and Banks, B. M., *New Eng. J. Med.,* 1952, v247, 880.

6. Flood, C. A., Jones, B., Rotton, W. M., and Schwartz, H., *Gastroenterology,* 1953, (April issue).

7. Segal, H. L., *J. N. Y. Acad. Sci.,* in press.

8. Maurer, W., and Zimmer, A., *Münchener Med. Wochenschrift,* 1952, v94, 1072.

9. Kelsey, F. E., and Geiling, E. M. R., *J. Pharm. and Exp. Therap.,* 1942, v75, 183.

Received May 20, 1953. P.S.E.B.M., 1953, v83.

Molybdenum Toxicity in *Lactobacillus leichmannii.* (20392)

LOUISE J. DANIEL AND LOUISE F. GRAY. (Introduced by L. A. Maynard)

From the Department of Biochemistry and Nutrition, Cornell University, and the U. S. Plant, Soil and Nutrition Laboratory, Bureau of Plant Industry, Soils and Agricultural Engineering, A.R.A., Ithaca, N. Y.

Molybdenum has been shown to be toxic for cattle(1-3), rats(4-6), and rabbits(7). Neilands, Strong and Elvehjem(4) found that whole liver substance overcame molybdenum toxicity in rats on an alcohol-extracted casein diet. Since this diet would be somewhat deficient in vit. B_{12}, it was thought that this vitamin might be involved in the correction produced by whole liver. A microorganism requiring vit. B_{12}, *Lactobacillus leichmannii* 4797, was chosen to expedite a study to determine if molybdenum produced a growth inhibition reversible by vit. B_{12}.

Experimental. Lactobacillus leichmannii 4797 was obtained from the American Type Culture Collection. The medium used and the procedure followed was that described by Peeler, Yacowitz and Norris(8), with the

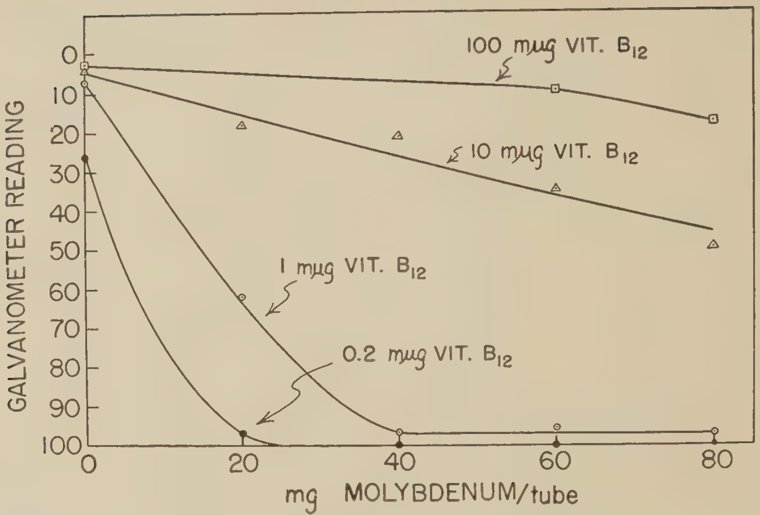


FIG. 1. Inhibition of growth of *Lactobacillus leichmannii* by molybdenum, and its reversal by vit. B_{12}. An 18-hr turbidimetric assay was used; a galvanometer reading of 100 represents no growth.

modifications noted. Each assay tube contained a final volume of 10 ml. When sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) was added to the medium, a yellow color was produced on steaming. The intensity of this color varied with the amount of molybdenum present. By adding each component of the basal medium separately to the molybdate solution, cysteine was found to be responsible for the color produced on steaming at pH 5.5, the pH of the original medium. As the pH was increased, less color was produced. However, pH 6.0 was the highest at which good growth of the organism could be obtained. Henceforth, the medium was modified by omitting cysteine, adjusting all supplements and the medium to pH 6.0, and by adding the molybdate solution after Seitz filtration to the sterile tubes. Either a 16-20-hour turbidimetric or a 40-48-hour titrimetric assay was employed.

Results. The first experiment was designed to establish the levels of molybdenum required to inhibit the growth of *Lactobacillus leichmannii*. Four levels of molybdenum were used, *i.e.*, 20, 40, 60 and 80 mg per tube in combination with several levels of vit. B_{12}. The results of this study are given in Fig. 1. Large amounts of molybdenum were required to inhibit the growth of this organism under the conditions stated, and relatively small quantities of vit. B_{12} prevented the inhibition. The relationship between molybdenum and vit. B_{12} appears to be competitive in nature, since as more molybdenum was added more vit. B_{12} was required to reverse its effect. Cobalt included at levels equivalent to the cobalt added as vit. B_{12} had no effect on molybdenum inhibition. Thus, the action of vit. B_{12} was apparently not due to cobalt *per se*.

The effect of cysteine on molybdenum toxicity was next investigated because of the earlier observation that cysteine and molybdate produced a yellow colored solution indicating possible complex formation. Levels of molybdenum, vit. B_{12} and cysteine were used in various combinations. Data representative of some of the results obtained are presented in Fig. 2. The addition of cysteine effectively reduced the inhibition caused by molybdenum. Methionine and cystine were then tried in combination with vit. B_{12} to learn if these compounds would have a similar effect; neither amino acid counteracted the inhibition caused by molybdenum.

Since cysteine is a reducing agent, it was of interest to determine if other reducing agents would decrease this inhibition by molybdenum. Ascorbic acid, sodium thioglycollate, glutathione and ferrous sulfate were tried. Only glutathione responded in a manner similar to

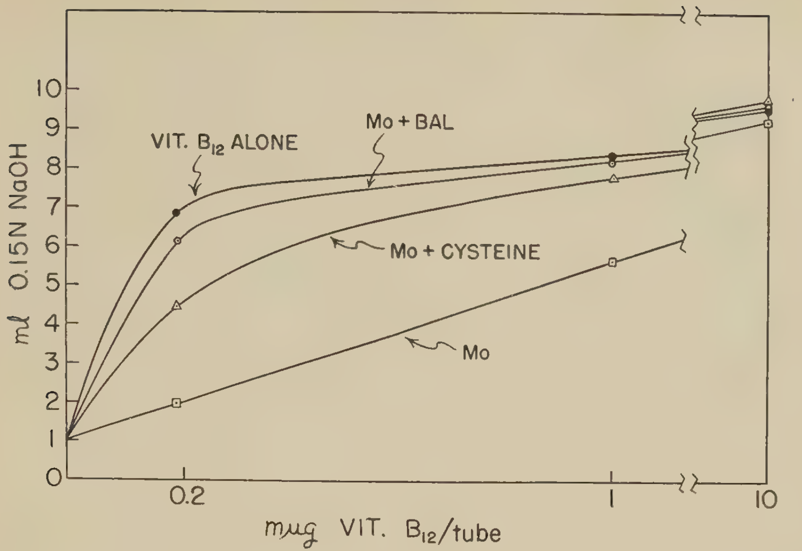


FIG. 2. Effect of cysteine and BAL on inhibition of *Lactobacillus leichmannii* by molybdenum at various concentrations of vit. B_{12}. A 41-hr titrimetric assay was employed. Molybdenum was added at 20 mg/tube, cysteine at 10 mg/tube, and BAL at 10 mg/tube. Molybdenum plus cysteine values have been corrected for growth stimulation caused by cysteine itself.

cysteine. The reducing property *per se* was apparently not the requirement for preventing molybdenum toxicity. The fact that both effective substances are sulfhydryl compounds suggested the study of 2,3-dimercaptopropanol, British antilewisite (BAL). A titrimetric assay was conducted in this instance because BAL produced a deep red color when added to the medium. The data for the BAL study are given in Fig. 2. BAL at 10 mg per tube was as effective as cysteine at 20 mg per tube in reversing the molybdenum inhibition.

Discussion. The mechanism by which molybdenum inhibits the growth of *Lactobacillus leichmannii* is unknown. Two possible explanations are: 1) The integrity of the sulfhydryl groups of certain enzymes may be destroyed. 2) Molybdenum may catalyze the hydrolysis of organic phosphate bonds of some essential metabolites.

The action of molybdenum on the sulfhydryl enzymes could be explained by the formation of chelates or mercaptides, or by the oxidation of the sulfhydryl group to the disulfide bond. The latter explanation is probably not valid, since reducing agents *per se* did not counteract the inhibition.

On the other hand, molybdenum has been shown to increase the hydrolysis of organic phosphate bonds(9,10), especially the high-energy bonds of adenosine triphosphate, acetyl phosphate, and creatine phosphate(9). Thus, molybdenum may decrease the effective concentration of compounds containing high energy phosphate bonds and hence inhibit growth.

The protective effect of cysteine, glutathione and BAL was probably due to the formation of mercaptides with molybdenum, thus reducing the amount of this metal available for its toxic action. Thiol compounds have long been known to reverse the inhibitory effect of mercaptide-forming agents on sulfhydryl enzymes. Barron and Kalnitsky(11) have found that in metal poisoning of succinoxidase, BAL was decidedly better than glutathione in reducing the inhibition. Since dithiols form more stable mercaptides than do monothiols, this would explain the greater effectiveness of BAL over cysteine or glutathione in reversing molybdenum toxicity.

The function of vit. B_{12} in reducing the inhibition of molybdenum is less readily explained. Several possible explanations are as follows: 1) Vit. B_{12} may be a part of a prosthetic group of an enzyme that is joined to the protein through a sulfhydryl group. 2) This vitamin may be maintaining the sulfhydryl enzymes in a reduced state as proposed by Dubnoff(12,13). 3) Vit. B_{12} may be concerned with the maintenance or production of high-energy phosphate bonds.

Summary. 1. Molybdenum at relatively high concentrations inhibited the growth of *Lactobacillus leichmannii.* 2. This inhibition was prevented or reduced by additions of vit. B_{12}, cysteine, glutathione or BAL. 3. Reducing agents *per se* had no effect on the inhibition. 4. The possible interactions of molybdenum, vit. B_{12}, and sulfhydryl compounds on the growth of the microorganism have been discussed.

---

1. Ferguson, W. S., Lewis, A. H., and Watson, S. J., *J. Agr. Sci.*, 1943, v33, 44.
2. Britten, J. W., and Goss, H., *J. Am. Vet. Med. Assn.*, 1946, v108, 176.
3. Comar, C. L., Davis, G. K., and Singer, L., *J. Biol. Chem.*, 1948, v174, 905.
4. Neilands, J. B., Strong, F. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1948, v172, 431.
5. Gray, L. F., and Ellis, G. H., *J. Nutrition*, 1950, v40, 441.
6. Comar, C. L., Singer, L., and Davis, G. K., *J. Biol. Chem.*, 1949, v180, 913.
7. Arrington, L. R., and Davis, G. K., *J. Animal Sci.*, 1952, v11, 756.
8. Peeler, H. T., Yacowitz, H., and Norris, L. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 515.
9. Weil-Malherbe, H., and Green, R. H., *Biochem. J.*, 1951, v49, 286.
10. Lutwak, L., and Sacks, J., *J. Biol. Chem.*, 1953, v200, 565.
11. Barron, E. S. G., and Kalnitsky, G., *Biochem. J.*, 1947, v41, 346.
12. Dubnoff, J. W., *Arch. Biochem.*, 1950, v27, 466.
13. ———, *Arch. Biochem. Biophys.*, 1952, v37, 37.

Received May 21, 1953. P.S.E.B.M., 1953, v83.

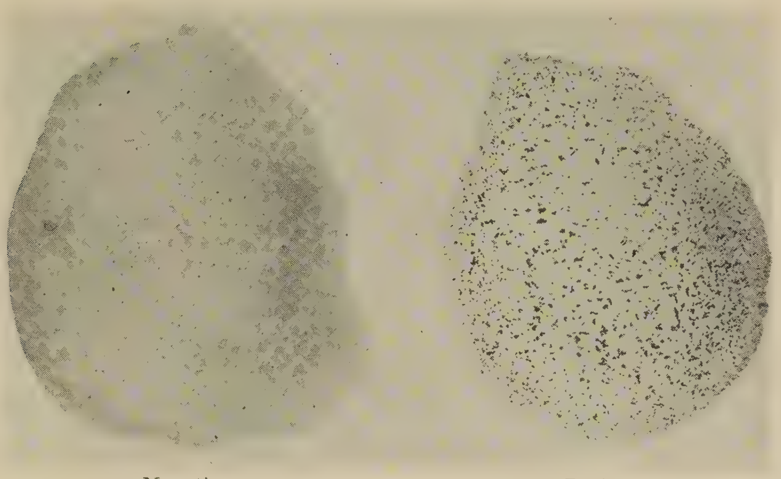
Leptospiral Macroscopic Agglutinating Antigens (20393)

WARREN G. HOAG, WILLIAM S. GOCHENOUR, JR., AND ROBERT H. YAGER.
From the Veterinary Division, Army Medical Service Graduate School, Washington, D. C.

The laboriousness of microscopic agglutination technics for the demonstration of leptospiral antibodies has severely limited their use in the laboratory diagnosis of leptospirosis. The search for simpler and more rapid methods, and for more stable antigens, has led to the development of a number of leptospiral macroscopic agglutinating antigens(1-4), but experience with antigens of these types has not been wholly satisfactory. Their sensitivity, stability, and specificity were not of the same order as that of the more widely used microscopic agglutinating antigens(5,6). Further, visible reactions were often granular in character and difficult to detect. Dye precipitation in stained antigens frequently confused interpretation. The potential usefulness of leptospiral macroscopic agglutinating antigens, however, warranted further studies directed toward overcoming these deficiencies. The influence of electrolytes upon stability of bacterial suspensions, and upon antigen-antibody reactions, has repeatedly been demonstrated(7-9). In particular, the sensitivity of bacterial agglutinating antigens has been shown to be markedly affected by changes in pH and ionic strength. Information about the various optimal conditions for leptospiral agglutinating reactions has not been reported in the literature available to the authors, so that initial studies were directed toward their determination. The adsorption of leptospiral agglutinating antigens onto inert particles had likewise not previously been reported, and it appeared reasonable to determine whether or not this procedure might result in the formation of more readily discernible aggregates in the agglutination reaction.

Macroscopic agglutinating antigens were prepared from nine leptospiral serotypes. The sensitivity, specificity, and stability of these antigens were investigated with immune sera prepared in rabbits and with sera from cases of human, equine, bovine, porcine, and canine leptospirosis.

Methods. 1) *Propagation of Leptospires.*



Negative Positive

FIG. 1. Positive and negative macroscopic agglutinating reactions.

Strains representing nine leptospiral serotypes were cultivated in Stuart's liquid medium(10) at 30°C for 10 to 14 days. The organisms were harvested at their maximum population density as determined by darkground microscopic examination. Leptospires were recovered by centrifugation at 43,300 x g for 45 minutes in a Spinco Model L refrigerated centrifuge, twice washed in one-tenth the original culture volume of distilled water, and again resuspended to one-tenth the original culture volume in distilled water. 2) *Standardization of Leptospiral suspensions.* Previous studies with freeze-dried leptospires(11) indicated optimal agglutinating antigen densities to be of the order of 0.5 mg of dried leptospires per ml. Such suspensions had 24.5% transmission at a wavelength of 600 mu on the Coleman Model 6A Spectrophotometer. This turbidity was also found optimal for non-freeze-dried leptospiral agglutinating antigen suspensions. Accordingly, the suspensions of washed leptospires, obtained above, were standardized to this transmission by the addition of distilled water. The standardized suspensions were incubated in the water bath at 56°C for four hours to kill the organisms. 3) *Determination of optimal pH.* Stained 0.1 M sodium acetate buffer solutions at pH 3.2, pH 4.7, pH 5.7 and pH 6.2, each containing 0.05 g of Crystal Violet (94% dye content) per 30 ml, were prepared. 0.2 ml of the buffers were added to 5.0 ml aliquots of the heat-killed leptospiral suspension. Serial 2-fold dilutions of homologous immune serum in distilled water were prepared through the range 1:5 through 1:2560. Equal volumes (ca 0.03 ml) of serum dilution and of antigen were mixed on a glass plate and incubated at room temperature for 10 minutes. Observations were then made with the unaided eye and reactions recorded as complete (+), incomplete (±), trace (T), or absent (–). The antigen adjusted to pH 5.7 was completely agglutinated by a 1:640 dilution of serum whereas antigens adjusted to pH 3.2, 4.7 and 6.2 resulted in endpoints of 1:20, 1:160 and 1:160 respectively. Based on these results, antigens were subsequently adjusted to pH 5.7. 4) *Determination of optimal ionic strength.* Stained acetate buffers at pH 5.7 with the same dye content as above were prepared at molarities of 0.025, 0.05, 0.10, 0.15 and 0.20. Each buffer in 0.2 ml quantity was added to 5.0 ml of heat-killed leptospiral suspension. These buffered suspensions were used as antigens in titration of homologous immune serum as described above. Antigens prepared at molarities of .05 and .10 were completely agglutinated at a serum dilution of 1:640 while molarities of 0.25, .15 and .20 gave endpoint titers of 1:160. Antigen prepared with 0.1 M stained acetate buffer at pH 5.7 was then used in the examination of homologous serum dilutions prepared in distilled water and in 0.85% NaCl solution. Results reveal the endpoint agglutinating titers of the serum diluted in distilled water and saline to be 1:640 and 1:160 respectively. 5) *Adsorption onto cholesterol platelets.* To each 5.2 ml of the buffered antigen was added 0.1 ml of a 1% solution of cholesterol (Pfanstiehl ash-free) in absolute ethanol. The cholesterol was added slowly with vigorous agitation of the flask containing the antigen. No effort was made to control the size of the platelets critically. The resultant precipitate was dispersed by shaking. 1.0 ml of glycerol was then added and the mixture again shaken. Comparison of cholesterolized and non-cholesterolized antigens in the examination of immune sera showed the former to possess approximately 4 times the sensitivity of the latter. 6. *Serologic technics.* a) *Macroscopic agglutination.* Serial 4-fold serum dilutions from 1:2.5 through 1:2560 were prepared in distilled water. A glass plate, ruled in one inch squares, was employed in a test box having oblique illumination, a black non-reflective background, and a hinged cover. One drop (ca 0.03 ml) of each serum dilution to be examined was placed on a separate ruled square. An equal quantity of antigen was added to each serum dilution; the antigen and serum dilution were mixed and spread over an area approximately 2 cm in diameter. Further mixing was assured by gently rocking the glass plate for the first minute of the test period and again at 3-minute intervals until the end of the 10-minute period. Agglutination was observed with the unaided eye and recorded as complete, incomplete or absent. Titers were expressed as the reciprocal of the highest dilution in which complete agglutination was observed. Inhibition in the region of antibody excess was frequently encountered in the examination of high titer sera, and, for this reason, all sera to be examined were carried through the dilution schema mentioned above. b) *Microscopic agglutination-lysis.* Parallel examination of serums with this method were conducted as described elsewhere(12).

Results. Cross agglutination reactions. Antisera prepared in rabbits against 18 leptospiral serotypes were examined with the 9 leptospiral macroscopic agglutinating antigens prepared. The results of these examinations are shown in Table I. Essentially similar cross reactions are observed in the agglutination-lysis antigen-antibody reactions(13). There is, however, somewhat greater serotype specificity shown by the macroscopic agglutinating antigens.

TABLE I. Macroscopic Cross-Agglutination Patterns of Leptospiral Serotypes.

Antiserums	Antigens								
	4	5	6	8	11	12	13	15	18
1. *L. andaman*									
2. *semerang*									
3. *hyos*									
4. *djatzi*	T								
5. *pomona*		T	4	16		16			4
6. *djasiman*		16	T	16			16		16
7. *sentot*			16						
8. *autumnalis,* Akiyami A				T			16		
9. *autumnalis,* FBF				16					
10. *australis,* A, Ballico									
11. *ballum*					T		16		
12. *Canicola,* Ruebush						T	16		16
13. *icterohemorrhagiae,* Wijnberg							T		16
14. *australis* B, Zanoni							16		
15. *grippotyphosa* Moscow V								T	
16. *alexi*									4
17. *medanensis*									
18. *pyrogenes, Salinem*									T

T = Titer of homologous strain; 4 = ¼ titer; 16 = 1/16 titer.

Comparison of macroscopic agglutination examinations with microscopic agglutination-lysis examinations. Six hundred and twenty-three bovine, 47 porcine, and 50 canine sera were examined by both macroscopic agglutination and microscopic agglutination-lysis technics. The same strains of the same serotypes of leptospires were employed as antigens with both technics. The results of these examinations are summarized in Table II. The table discloses that the degree of correlation between these methods at significant antibody levels is of the order of 70.0% Correlation approaches 80.0% when sera with high antibody levels are examined.

Serial serum specimens from proved cases of Leptospirosis pomonae were examined. The results are summarized in Table III. Significant rises and falls in antibody levels are demonstrable with this technic.

Stability. No loss of sensitivity has been

TABLE II. Examination of Bovine,* Porcine,* and Canine† Sera by Macroscopic and Microscopic Agglutination Technics.

Microscopic agglutination	Macroscopic agglutination titer																					No. sera		
	0			5			20			80			320			1280			5120					
titer	‡B	P	C	B	P	C	B	P	C	B	P	C	B	P	C	B	P	C	B	P	C	B	P	C
102400	13	0	0	3	0	0	4	0	0	8	0	0	20	4	0	13	0	0	4	0	0	65	4	0
25600	5	0	0	13	0	0	3	2	0	5	2	0	4	0	0	9	0	0	0	0	0	39	4	0
6400	11	0	0	22	1	0	6	3	0	1	1	2	2	1	0	1	0	0	2	0	0	45	6	2
1600	14	0	0	41	2	2	8	3	8	5	1	2	0	0	0	0	0	0	0	0	0	68	6	12
400	41	2	1	23	1	5	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	66	3	7
100	39	4	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	41	4	2
0	291	20	27	5	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	299	20	27
No. sera	414	26	28	109	4	9	26	8	9	19	4	4	26	5	0	23	0	0	6	0	0	623	47	50

* *L. pomona*, S-91 (Gochenour, W. S., Jr., Johnston, R. V., Yager, R. H., and Gochenour, W. S., *Am. J. Vet. Res.*, 1952, v13, 158) employed as antigen with both technics.

† *L. canicola* Ruebush (Randall, R. and Cooper, H. K., *Science*, 1944, v100, 133) employed as antigen with both technics.

‡ B = Bovine sera; P = Porcine sera; C = Canine sera.

TABLE III. Examination of Serums from Cases of Leptospirosis Pomonae.

Source	Specimen	Macroscopic agglutination titer	Microscopic agglutination titer
Human	1st	0	0
	2nd	320	200
Bovine	1st	80	1600
	2nd	1280	102400
Exp. equine	P*	0	0
	69 day	80	500000
	329 "	5	800
" rabbit	P	0	0
	7 day	20	800
	14 "	80	6400
	21 "	1280	6400

* P = Pre-infection.

observed following storage at 5°C for 15 months. Of interest, however, is the observation that these antigens do not attain their maximum sensitivity until approximately 48 hours after preparation.

Discussion. The optimal conditions for specific agglutination in this system appeared to occur at pH 5.7 and at ionic strengths less than 0.10. Preparation of the antigen as described, and the use of distilled water as a diluent for the sera examined, afforded a convenient approximation to this condition. Macroscopic agglutinating antigens prepared in this manner can play a useful role in the laboratory diagnosis of leptospirosis. The simplicity of the technic of the test, and the stability of the antigens, recommend this procedure to the small laboratory. The lack of correlation between macroscopic agglutination and microscopic agglutination tests in the examination of low titer sera from cattle is a limiting factor in the employment of antigens of this type for serological surveys of bovine leptospirosis. The high antibody levels attained by cattle during the acute phase of leptospiral infection, however, is such that these two technics should be equally reliable for the diagnosis of acute leptospirosis. Perhaps the greatest value of these antigens lies in their utilization in field surveys of outbreaks of acute leptospirosis in domestic animals. Here, where time may be of the essence in the application of measures to limit and control infection, rapid diagnosis of acute cases may be of paramount significance.

Summary. A method for the preparation of macroscopic leptospiral agglutinating antigens is described. Antigens of this type have been prepared from 9 leptospiral serotypes. Cross reaction patterns are essentially comparable to those observed in the agglutination-lysis technic. Antigens so prepared are sensitive, specific, and stable under protracted periods of storage, and produce easily discernible reactions in the presence of antibodies.

The authors acknowledge, with thanks, the advice of Dr. C. W. Hiatt and the technical assistance of Mr. LaRue B. Evans in the performance of microscopic agglutination-lysis examinations.

---

1. Starbuck, E. B., and Ward, T. G., *J. Inf. Dis.*, 1941, v70, 88.
2. Pot, A. W., *Lancet,* 1936, v1, 1290.
3. Gardner, A. D., and Wylie, J. A. H., *Lancet,* 1946, v1, 955.
4. Brown, H. C., *Brit. Med. J.*, 1939, v2, 1138.
5. Esseveld, H., Thesis, Amsterdam, 1937.
6. Van Thiel, P. H., *The Leptospiroses,* Universitaire Pers, Leiden, 1948, 31.
7. Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity,* 3d ed., Williams and Wilkins Co., Baltimore, 1946, 211.
8. Northrop, J. H., and DeKruif, P. H., *J. Gen. Physiol.,* 1922, v4, 639.
9. *Ibid.,* 1922, v4, 655.
10. Stuart, R. D., *J. Path. and Bact.,* 1946, v58, 343.
11. Unpublished data.
12. Gochenour, W. S., Jr., Yager, R. H., Wetmore, P. W., and Hightower, J. A., *A. J. P. H.,* 1953, v43, 405.
13. Gochenour, W. S., Jr., and Yager, R. H., *Proc. A. V. M. A.,* 1952, 178.

Received May 22, 1953. P.S.E.B.M., 1953, v83.

Relative Biological Effectiveness of Radium and Other Alpha Emitters in CF No. 1 Female Mice. (20394)

MIRIAM P. FINKEL.

From the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.

Estimates of permissible levels of radioactive isotopes in man depend, in part, upon a comparison of the radiotoxic hazards of these elements and of radium. Radium has become the common denominator for experimental animals and man because of the information available from the study of individuals who were exposed accidentally or who were intentionally treated with it. The data at hand from a series of experiments in progress make possible comparisons a) of the effects of radium and some other alpha emitters on the survival of mice, and b) of their carcinogenic potentialities for the mouse skeleton.

Procedures. The dose ranges of the radioisotopes used are presented in Table I. Radium 226, polonium 210, and thorium 228 were injected as chlorides; plutonium 239 (plus 4) and the 2 uranium isotopes were given as nitrates. Except for uranium 232 and thorium 228, the doses were selected to range from rapidly lethal levels to levels below tolerance. The materials, in isotonic salt solution at pH 2-4, were injected into a lateral tail vein of CF No. 1 female mice that were 9-10 weeks old. Complete protocols and details concerning the handling of these duration-of-life experiments will be presented when the studies are finished. Briefly, all animals were autopsied, tissues were taken for histological examination, and roentgenograms were prepared. Whenever possible, animals were sacrificed when moribund.

TABLE I. Materials and Range of Doses.

Isotope	Range of doses (μc/kg)	No. of animals
Radium 226	.6 –4170	675
Plutonium 239	.04– 50.4	765
Polonium 210	.46– 100	645
Uranium 233	.1 – 100	675
” 232	.1 – 10	135
Thorium 228	6.6 – 360	75
Controls		450
Total		3420

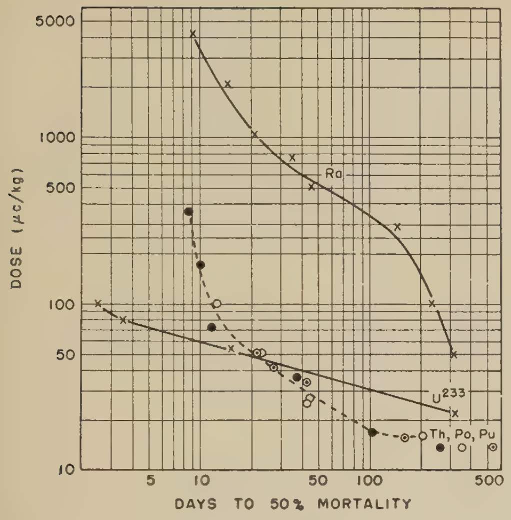


FIG. 1. Relationship between isotope-dose and time to 50% mortality.

Results. Time to 50% mortality. The effectiveness of the isotope-doses as measured by the time required to kill 50% of the animals is presented in Fig. 1. A single curve expresses the thorium, polonium, and plutonium data since the values were close over the range where the doses overlapped. The differences in the slopes of the 3 curves during the first 15 days after injection can be explained on the basis of the μc:μg ratio of the various radio-isotopes, *i.e.*, whether death was due to irradiation, which kills relatively slowly, or to chemical toxicity, which can kill rapidly. Since one μc of thorium 228 is equivalent to only 0.0012 μg, death was due to irradiation alone. This is borne out by the slope of the curve, where it can be seen that 72 μc/kg was almost as effective as 360 μc/kg. On the other hand, 100 μc/kg of uranium 233 resulted in death almost immediately. In this case the amount of uranium injected (1 μc = 100 μg) was above the chemically lethal dose (3). The curve describing the radium data up to the fifteenth post-injection day is intermediate in slope, and it is likely that at these levels there was a chemical as well as a radiation effect. Because of these considerations, comparisons of μc dosages that resulted in 50% mortality in less than 20 days are not justified. In addition, the curves past 150 days have little significance since the control populations reached 50% mortality at 400-450 days. From 20 to 100 days the LD$_{50}$ per given time for radium remained approximately 20 times greater than that for thorium, polonium, and plutonium and varied from 23 to 11 times greater than that for uranium 233. In other words, these alpha emitters killed CF No. 1 female mice approximately 20 times as efficiently as radium at levels high enough to halve the population in 20 to 100 days.

Life expectancy. Fig. 2 illustrates the expectation of life expressed as the percentage of the life expectancy of the control populations immediately after injection. Higher doses of radium than of any of the other materials were required to decrease by 50%

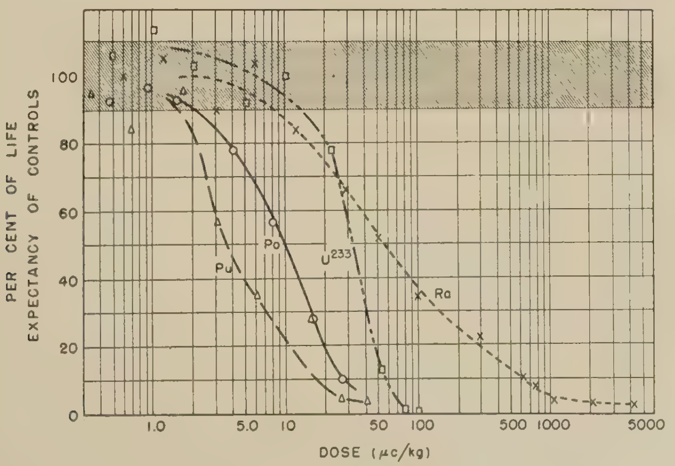


FIG. 2. Relationship between expectation of life and dose proportionate to the expectation of life of the control populations (100% ± 2 S.D. of mean).

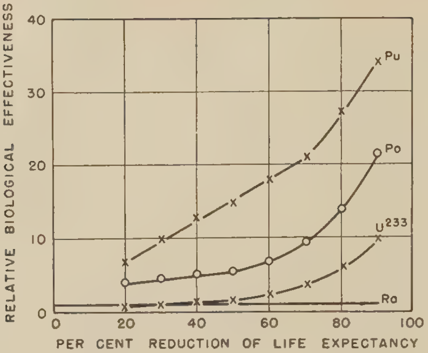


FIG. 3. Biological effectiveness relative to radium in reducing expectation of life.

or more the average number of days lived. These curves were used in the preparation of Fig. 3, which presents the effectiveness of plutonium, polonium, and uranium 233 relative to radium in reducing the expectation of life. The greater the effect, the greater the divergence between radium and the others. For example, the factors are 34 (Pu), 21 (Po), and 10 (U233) for 90% reduction and 8, 4, and 0.75, respectively, for 20% reduction. It is the minimal effect produced by very low doses that must be known in order to establish permissible levels in man. Unfortunately, these are the data that are most difficult to obtain. The values derived from the curves in Fig. 2 for 10% reduction are meaningless in view of the deviation among the control populations. However, if the curves in Fig. 3 are extended toward zero reduction, those for plutonium and radium meet at zero but that for polonium does not go below three. It appears, then, that as ineffectual levels are approached, polonium remains three times as effective as radium in reducing the expectation of life.

Malignant bone tumors. The carcinogenic property of bone-seeking radio-isotopes furnishes another criterion by which the hazards of different materials can be compared. Radium, plutonium, and uranium localize in bone while polonium and thorium do not. Although the incidence of malignant bone tumors after the injection of some levels of polonium was higher than that among the control animals, it was much lower than that following the injection of comparable doses of the bone-seekers. The data presented here are tentative in that every case of possible malignancy has not yet been verified histologically; only the positively diagnosed tumors have been included.* However, it is expected that the final tabulation of the number of animals with malignant bone tumors will not vary greatly from that now at hand.

Within each experimental unit the expectancy of bone tumors remained relatively constant after the first malignancy appeared. A single figure for each dose-level group was obtained by averaging these daily tumor expectancies, and the data are plotted in Fig. 4. The expectancy value among the control animals was 3.3%. The unfinished, steeply declining line following the peaks of the curves indicates that at the next dose the tumor expectancy was zero or close to zero, either because the animals did not live long enough or because they were so debilitated that tumors did not appear. Such a peak was not obtained with the doses of uranium 232 used. On the assumptions that the slope of this curve is correct and that uranium 232 is no more toxic to the entire organism than ura-

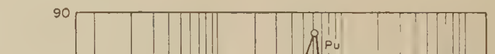


FIG. 4. Probability of developing a malignant bone tumor after the injection of various isotope-doses if surviving the latent period.

---

* The author is indebted to Dr. Donald Russ for examination of many of the histological preparations.

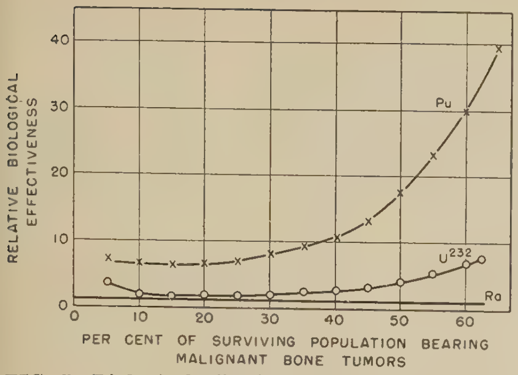


FIG. 5. Biological effectiveness relative to radium in producing malignant bone tumors.

nium 233, a maximum probability of tumor incidence of approximately 80% would be expected after the injection of 22 μc/kg of uranium 232. The difference in tumor producing effect between these two isotopes of uranium is probably due to the fact that uranium 232 (5.3 mev ∝) decays to the 1.9 year thorium 228 (5.4 mev ∝), while uranium 233 (4.8 mev ∝) decays to the 7000 year thorium 229 (4.85 mev ∝).

In Fig. 5 the radium values have been converted to unity and those for plutonium and uranium 232 plotted accordingly. As was noted previously, the effectiveness of the more potent isotopes relative to radium increases as the severity of the response increases. In this case, however, the curves describing the plutonium and uranium 232 data show no indication of decreasing to zero. At present the best estimate that can be made of their relative effectiveness in producing malignant bone tumors in CF No. 1 female mice at levels approaching those that are ineffectual is 7 (Pu):2 (U233):1(Ra).

Discussion. The relative biological effectiveness of radium and the other alpha-emitting isotopes studied varies with the criterion of effect and with the degree of the response being measured. Many factors enter into considerations of the reasons for these deviations, such as the biological half-time of the various radio-isotopes, their distribution, and the radio-sensitivity of the tissues in which they are most heavily concentrated. Boyd *et al.* noted similar variations in their studies which compared the effect of polonium and plutonium with that of radium on the median survival time in rats(1).

From the data on CF No. 1 female mice presented here, the best figures that can be derived for near-tolerance levels relative to radium are 7 for plutonium, 3 for polonium, 2 for uranium 232, and 1 for uranium 233. The data concerning time to 50% mortality and life expectancy are complete, but other considerations must enter into a final evaluation of the efficiency with which these radioisotopes produce bone malignancies, *e.g.*, the length of the latent period and the number of tumors per animal. The available data suggest that the length of the latent period is independent of the dose level, but it may vary with the isotope. However, an eventual analysis in terms of the total number of primary malignancies per group may be of greater value than the present analysis, which is based on the total number of animals with tumors per group. Two or more bone tumors per animal were occasionally found among those that received some doses of radium or uranium, but multiple tumors were the rule after the most effective doses of plutonium.

These tentative factors will be subject to revision as other organs and tissues are studied. For example, in view of the relatively high, early concentration of polonium in the ovary(2) and of uranium in the kidney(3-5), these may prove to be the critical organs in establishing tolerance levels for these elements.

Summary. 1. Plutonium 239, polonium 210, uranium 233, and thorium 228 were 20 times as effective as radium 226 in killing 50% of a population of CF No. 1 female mice in 20 to 100 days after intravenous administration. At levels approaching those that produced no effect, polonium was 3 times as effective, plutonium was equally effective, and uranium 233 was slightly less effective than radium in decreasing the expectation of life. The ratios relative to radium of the proportion of mice that developed malignant bone tumors after the latent period were 7:1 for plutonium and 2:1 for uranium 232. Uranium 233 and polonium were less effective than radium in this instance. 2. On the basis of these criteria, the best present estimate of the relative biological effectiveness at near-tolerance levels in CF No. 1 female mice is 7 (plutonium 239):3, (polonium 210):2, (uranium 232):1 uranium 233):1 (radium 226).

Grateful acknowledgment is made to Mrs. H. Scribner for her assistance in all phases of the work and to Mrs. D. Bansch, Miss J. Lestina, and Mrs. P. Sieck for their technical aid.

---

1. Boyd, G. A., Silberstein, H. E., Fink, R. M., Frenkel, A., Minto, W. L., Metcalf, R. G., Casarett, G., Suter, G. M., Williams, A., and Tiedeman, D. V., Chap. 7 and 8, in *Biological Studies with Polonium, Radium, and Plutonium,* Ed., R. M. Fink, National Nuclear Energy Series, VI-3, McGraw-Hill, New York, 1950.

2. Finkel, M. P., Norris, W. P., Kisieleski, W. E., and Hirsch, G. M., *Am. J. Roentgenol. Radium Therapy Nuclear Med.,* in press.

3. Hodge, H. C., *Introduction to Pharmacology and Toxicology of Uranium Compounds,* Ed., C. Voegtlin and H. C. Hodge, Vol. I, National Nuclear Energy Series VI-1, McGraw-Hill, New York, 1949.

4. Kisieleski, W. E., Faraghan, W. G., Norris, W. P., and Arnold, J. S., *J. Pharmacol. Exp. Therap.,* 1952, v104, 459.

5. Tannenbaum, A., *Toxicology of Uranium,* National Nuclear Energy Series, IV-23, McGraw-Hill, New York, 1951.

Received June 3, 1953. P.S.E.B.M., 1953, v83.

Absorption of Cholesterol-4-C^{14} in Rats Fed Mixed Soybean Sterols and β-Sitosterol.* (20395)

H. H. HERNANDEZ, D. W. PETERSON, I. L. CHAIKOFF, AND W. G. DAUBEN.

From the Department of Physiology, School of Medicine, Department of Poultry Husbandry, College of Agriculture, and Department of Chemistry, University of California.

The hypercholesteremia and atherosclerosis observed in the cholesterol-fed chicken can be prevented by the addition of soybean sterols to the diet. To explain this phenomenon the suggestion has been made that the plant sterols interfere with the absorption of cholesterol(1,2). In the experiments presented here we have tested this hypothesis by feeding rats C^{14}-labeled cholesterol together with either β-sitosterol or soybean sterols and determining the recovery of the C^{14} in thoracic duct lymph.

Experimental. Rats of the Long-Evans strain weighing 250-300 g were used. They were maintained on a stock diet with the following composition: ground whole wheat, 64.3%; casein, 14.3%; skim milk powder, 6.8%; NaCl, 0.7%; $CaCO_3$, 1.4%; Wesson Oil, 11.5%; fish oil, 1%; and a trace of KI. Forty-eight hours before the experiment all food was removed from the cages, but for the 24 hours just preceding the operation the animals again had access to the stock diet. This feeding procedure ensured the presence of food in the intestinal tract, and consequently a copious lymph flow, at the time the cannulae were inserted into the thoracic ducts. The procedure used for collection of thoracic duct lymph has been described elsewhere(3). Rats with poor lymph flow were discarded at the time of operation. Immediately after the cannulation a test meal containing labeled cholesterol (Table I) was administered by stomach tube. During the rest of the experiment the rats had access to the stock diet and 0.9% saline for drinking purposes. The synthesis of cholesterol-4-C^{14} used in this study was described earlier(4). *Total C^{14} content of lymph* samples was determined as described elsewhere(5). Free and total cholesterol was determined by the procedure of Sperry and Webb(6). The C^{14} content of the cholesterol digitonide was determined by dissolving it in methanol and a suitable aliquot mounted on an aluminum plate for counting.

Results and discussion. The effects of mixed soy sterols and of β-sitosterol upon the recoveries of C^{14} in thoracic duct lymph were studied. The design of the 2 experiments is shown in Table I. It is clearly shown there that the simultaneous administration of C^{14}-cholesterol with either mixed soy sterols on

---

* Aided by grants from the U. S. Public Health Service and the Life Insurance Medical Research Fund.

TABLE I. Recovery of Enterally Administered Labeled Cholesterol in Lymph of 20 Rats.

Exp.	Test meal	% administered C^{14} recovered in thoracic duct lymph in 48 hr	% lymph's C^{14}-cholesterol in esterified form
1	0.25 cc cottonseed oil followed by 4 mg C^{14}-cholesterol in 0.25 cc cottonseed oil	26	63
		21	39
		30	47
		18	41
		25	58
		30	50
		32	42
	4 mg mixed soy sterols* in 0.25 cc cottonseed oil followed by (‡)	6	39
		8	34
	25 mg mixed soy sterols* in 0.25 cc cottonseed oil followed by (‡)	0.6	
		5	11
2	0.25 cc cottonseed oil followed by (‡)	33	42
		37	59
		43	51
		34	47
	4 g β-sitosterol† in 0.25 cc cottonseed oil followed by (‡)	8	22
		7	25
	25 mg β-sitosterol† in 0.25 cc cottonseed oil followed by (‡)	1	20
		5	23
		1	41

* Reported to contain 95% sterols, 10-15% of which were stigmasterol and 75-80% sitosterols. We are indebted to Distillation Products Industries for the mixed soy sterols.

† We are indebted to Lederle Laboratories for the β-sitosterol.

‡ 4 mg C^{14}-cholesterol in 0.25 cc cottonseed oil.

β-sitosterol resulted in a pronounced decrease in the C^{14} recoveries in lymph. These results show that plant sterols interfere with cholesterol absorption in the rat. Tomkins, in this laboratory(7), has shown that hepatic cholesterogenesis from acetate in the rat is not influenced by feeding it a diet containing 2.5% mixed soy sterols and 5% cottonseed oil for 6 days. It therefore seems likely that plant sterols prevent hypercholesterolemia in birds fed cholesterol by interfering with the absorption of the cholesterol.

The data recorded in the last columns of Table I show the percentages of the labeled lymph cholesterol that were in esterified form. It would appear that the administration of the mixed soy sterol or of β-sitosterol decreased the percentages of the C^{14}-cholesterol in the esterified form. This decrease suggests that the plant sterols may act by interfering with esterification of cholesterol in its passage from the lumen of the intestine to lymph.

Summary. 1. The effect of plant sterols on cholesterol absorption was studied. Ring-labeled cholesterol was fed by stomach tube to rats into whose thoracic ducts cannulae had been inserted. 2. Simultaneous feeding of mixed soy sterols or of β-sitosterol with labeled cholesterol resulted in a pronounced reduction in the C^{14} recoveries in thoracic duct lymph. 3. Approximately 50% of C^{14}-cholesterol in lymph was esterified when the test meal contained only labeled cholesterol and cottonseed oil. Less of lymph's C^{14}-cholesterol was esterified when mixed soy sterols or β-sitosterol were added to test meal.

---

1. Peterson, D. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 143.

2. Peterson, D. W., Nichols, C. W., Jr., and Schneour, E. A., *J. Nutrition*, 1952, v47, 57.

3. Bloom, B., Chaikoff, I. L., Reinhardt, W. O., Entenman, C., and Dauben, W. G., *J. Biol. Chem.*, 1950, v184, 1.

4. Dauben, W. G., and Eastham, J. E., *J. Am. Chem. Soc.*, 1951, v73, 3260.

5. Siperstein, M. D., Chaikoff, I. L., and Reinhardt, W. O., *J. Biol. Chem.*, 1952, v198, 111.

6. Sperry, W. M., and Webb, M., *J. Biol. Chem.*, 1950, v187, 97.

7. Tomkins, G. M., unpublished observations.

Received June 3, 1953. P.S.E.B.M., 1953, v83.

Further Studies on Metabolism of Tissues from Thyroxine-Injected Rats.* (20396)

S. B. Barker and H. S. Schwartz.

From the Departments of Zoology, University of California at Los Angeles, and of Pharmacology, Medical College of Alabama and School of Dentistry, University of Alabama, Birmingham.

Barker and Klitgaard(1) reported the effects of thyroidectomy and of thyroxine injection on the oxygen consumption of various tissues excised from rats. Their results showed that liver, kidney, gastric mucosa and several types of muscle (including cardiac, skeletal, and diaphragmatic and gastric smooth muscle) exhibited a depressed metabolic rate following thyroidectomy and increases to various extents after injection of a single large dose of thyroxine. In contrast, brain, spleen and testis were not altered by either hypo- or hyperthyroidism.

The present study was designed to investigate some of the implications of this work, such as possible effects on the female gonads as well as on accessory reproductive structures of both sexes and on other exocrine glands of the digestive tract.

Methods. Since many of the tissues included here were found not to be controlled by the thyroid, repeated injections of a smaller dose of hormone were employed to make certain that maximal increases in metabolic rate of the animal were obtained. Two mg of L-thyroxine[†] were injected subcutaneously per kilo of body weight per day for 4 days and the animal sacrificed on the fifth. In general, procedures were as outlined previously(1). Each animal was anesthetized with ether, and as much blood as possible withdrawn by syringe from the abdominal aorta. The desired tissues were then rapidly excised and chilled in ice-cold Ringer solution. In some cases, animals were sacrificed without anesthesia by guillotine in order to avoid possible effects of ether. In the preparation of tissues for oxygen consumption studies, connective tissue was removed as completely as practicable, in addition to all extraneous material such as the coagulating glands and seminal fluid from the seminal vesicles. Liver, kidney cortex, salivary glands, thymus and spleen were sliced according to Deutsch(2). The thinnest portions from each hemi-diaphragm were used. Uteri and seminal vesicles were opened lengthwise, the prostate was cut several times, and appropriate quantities of each were taken. Lymph nodes, ovaries and brain were cut several times along different planes with scissors and the resulting rough mince employed. Oxygen constituted the gas phase. Except in the case of ovaries and thymus, where only small quantities of tissue were available, duplicate vessels were set up, using Krebs-Ringer phosphate solution with 100 mg % of glucose. All results are shown in terms of wet weight.

Results and discussion. Table I presents the means and standard deviations of the tissues studied representing euthyroid, thyroidectomized and thyroxine-injected thyroidectomized animals.

Liver, kidney and diaphragm were included as positive controls, since these have previously been found to respond consistently to the level of thyroid function(1). The decreases of 15-30% following removal of the thyroid gland and increases of 50-75% resulting from thyroxine injection (Table II) agree with results from this and other laboratories and furnish assurance that the animals actually were in the metabolic conditions desired.

Salivary gland, completely exocrine, and pancreas, mixed exo- and endocrine, both displayed changes in oxygen consumption indicating dependence upon thyroxine: approximately 20% decreases after thyroidectomy and a 34% increase during thyroxine injection from salivary gland, 51% with pancreas.

---

[*] This work was carried out during tenure of a Krichesky-Ponty Memorial Fellowship at the University of California.

[†] Material obtained through the courtesy of Glaxo Laboratories, Smith, Kline and French Laboratories and Baxter Laboratories, Inc.

TABLE I. Metabolism of Various Tissues in Euthyroid, Thyroidectomized and Thyroxine-Injected Thyroidectomized Rats.

	Euthyroid		Thyroidectomized		Hyperthyroid	
		mm³ O_2/mg/hr		mm³ O_2/mg/hr		mm³ O_2/mg/hr
Tissue	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.
Liver	12	1.26 ± .09	12	1.01 ± .07	12	1.64 ± .11
Kidney	12	3.72 ± .23	12	3.17 ± .13	12	4.68 ± .44
Diaphragm	12	.95 ± .08	11	.66 ± .07	11	1.14 ± .17
Salivary gland	8	2.31 ± .06	8	1.81 ± .08	8	2.43 ± .11
Pancreas	8	1.04 ± .04	7	.83 ± .07	8	1.25 ± .13
Lymph node	11	.87 ± .12	11	.85 ± .09	12	.92 ± .08
Thymus	12	1.09 ± .14	12	.99 ± .18	11	1.09 ± .15
Ovaries	6	1.14 ± .10	6	1.13 ± .11	6	1.04 ± .12
Uterus	6	.73 ± .09	6	.73 ± .10	6	.78 ± .06
Seminal vesicle	6	.77 ± .05	6	.75 ± .07	6	.75 ± .04
Prostate	6	1.52 ± .10	6	1.51 ± .16	6	1.54 ± .14
Brain	4	1.30	4	1.28	4	1.37
Spleen	4	1.42	4	1.35	4	1.48

TABLE II. Change in O_2 Consumption of Various Tissues Produced by Thyroidectomy and by Thyroxine Injection.

	Thyroidectomy change		Thyroxine inj. change	
	%	P	%	P
Liver	—19.8	<.01	+62.4	<.01
Kidney	—14.8	”	+47.6	”
Diaphragm	—30.6	”	+72.7	”
Salivary gland	—21.6	”	+34.3	”
Pancreas	—20.2	”	+50.6	”
Lymph nodes	— 2.3	>.1	+ 8.2	<.1, >.05
Thymus	— 9.2	”	+10.1	>.1
Ovaries	— .9	”	— 8.0	”
Uterus	.0		+ 6.8	”
Seminal vesicle	— 2.6	”	.0	
Prostate	— .7	”	+ 2.0	”
Brain	— 1.5		+ 7.0	
Spleen	— 4.9		+ 9.6	

These alterations are in keeping with similar ones exhibited by gastric mucosa, since Barker and Klitgaard(1) found a 28% decrease after thyroidectomy and a 60% peak increase in hyperthyroidism.

Lymph nodes (primarily cervical in origin) and thymus both constitute examples of reticulo-endothelial tissue. The former showed no significant variations, and the latter only a 10% change, of dubious significance considering the variations in individual values. In addition, such a change is far below the 35-75% alterations simultaneously produced in liver, kidney, diaphragm, salivary gland and pancreas.

Ovarian metabolism was not altered in the three states of thyroid function; thus they can be aligned with the male gonads, previously studied. A similar series of no metabolic responses was obtained with accessory sex structures of both sexes, including uterus, seminal vesicles and prostate. The "castration" effects of hypothyroidism on the female rat, involving prolonged diestrus(3) as well as increased sensitivity to administered estrogen (4), obviously cannot be explained on a purely metabolic basis.

Results are also shown on brain and spleen of a small number of animals in each status of thyroid function sacrificed without anesthesia. The data for brain metabolism are about 30% higher than those previously reported(1); this occurred because the brain mince previously was cleared of soluble materials by suspension in cold Krebs-Ringer solution and decantation. This process was omitted in the present series of experiments. Even with these different conditions, however, changes of only 1.5% and 7.0% from euthyroid values were produced by thyroidectomy and hyperthyroidism, respectively. In the case of spleen, the results were very close to those reported by Barker and Klitgaard.

A survey of Table II reveals a considerable number of tissues showing no metabolic relationship to thyroid hormone level, including lymph nodes, thymus, ovaries, uterus, seminal vesicles, prostate, brain, and spleen. Testis, studied previously, can be added. Using the t-test for statistical significance of the differences in Qo_2 between euthyroid and thyroidectomized and between thyroidectomized and hyperthyroid, every one of these tissues has a P in excess of 0.1, indicating a very low level of confidence in any differences present. Examination of the entire series shows that several tissues exhibited increases of 7 to 10% during thyroxine injection; it is quite possible that accumulation of many more data for each one would eventually produce statistically valid differences. The group of tissues would still, however, stand in contrast to the others with much more marked effects. The conclusion seems valid that brain, lymphoid tissue, gonads and accessory reproductive structures are not responsive to thyroid hormonal control of metabolism, in contrast to several kinds of muscle and glands producing digestive secretions.

Preliminary results obtained on the distribution of radioactive thyroxine‡ following a single injection indicate that the lack of metabolic stimulation by the hormone, where this situation prevailed, cannot be attributed to a lack of exposure of these tissues to the hormone. With an impressive list accumulating of tissues not responding to thyroxine, the question of mechanism of action of this material becomes ever more intriguing. If the primary effect is one of uncoupling of oxidative and phosphorylative reactions or a direct involvement of the hormone as a member of an oxidation-reduction system(5), marked variations must occur in how these processes respond in different tissues.

Summary. Salivary gland and pancreas have been added to the list of tissues whose energy metabolism is controlled by the thyroid gland. In contrast, lymph nodes, thymus, ovaries, uterus, prostate and seminal vesicles have metabolic rates unaltered (within 10%) by thyroid status.

1. Barker, S. B., and Klitgaard, H. M., *Am. J. Physiol.*, 1952, v170, 81.

2. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, Burgess Pub. Co., Minneapolis, 1949, pp. 110-111.

3. Nelson, W. O., and Wheeler, H. E., *Fed. Proc.*, 1948, v7, 85.

4. Langham, W., and Gustavson, R. G., *Am. J. Physiol.*, 1947, v150, 760.

5. Barker, S. B., *Physiol. Rev.*, 1951, v31, 205.

Received June 4, 1953. P.S.E.B.M., 1953, v83.

---

‡ Radioactive thyroxine synthesized by S. C. Wang, T. Winnick, S. Notrica, and R. J. Winzler from I^{131} allocated by the Atomic Energy Commission.

Effect of Cortisone on Diphtheria Intoxication and the Schick Test in Guinea Pigs.* (20397)

P. ROSENBAUM AND W. OBRINSKY. (Introduced by M. E. Wegman.)

From the Department of Pediatrics, Louisiana State University School of Medicine, New Orleans.

A study of the effect of cortisone on diphtheria intoxication in guinea pigs was carried out as part of an attempt to assess the value of cortisone in infections. Diphtheria toxin, rather than *C. diphtheriae*, was used in order to exclude complications which might have resulted from the interference of cortisone with host resistance to invading microorganisms(1-4).

The observation that ACTH appeared to alleviate the effects of the neurotoxin elaborated by the black widow spider(5) suggested that cortisone might be of benefit in diphtheria intoxication.

Material and methods. Male and female guinea pigs weighing approximately 300 g were used. They were kept in separate cages, fed cabbage and rabbit pellets, and offered both tap water and 1% KCl solution *ad lib.* The last was used to prevent cortisone-induced hypokalemia. Animals were inspected daily. They were weighed initially and at the time of death or at the end of 10 days in the event of

---

* Supported by a research grant from Playtex Park Corp. Diphtheria antitoxin and diphtheria toxin were supplied by Dr. J. M. Ruegsegger of Lederle and Co. Cortisone acetate was supplied by Dr. E. Alpert of Merck and Co.

TABLE I. Effect of Cortisone on the Schick Test.

	No. of animals		Time of max reaction (days)	Area of max reaction (cm²)	Healing time (days)
Controls	10	Avg	3.2	4.0	10.1
		Range	2–5	1–12	5–14
Cortisone-treated	7	Avg	2.4	5.2	12.7
		Range	1–3	0.25–8	5–20

TABLE II. Effect of Cortisone on Guinea Pigs Given 0.5 M.L.D. Diphtheria Toxin.

	No. of animals	No. of deaths	Avg initial wt (g)	Avg final wt (g)	Avg survival time of animals (days)
Controls	35	29	308	256	4.3
Cortisone-treated	35	27	307	248	5.0

their survival. All injections other than Schick tests were given subcutaneously in the lumbar region. There were 3 experimental groups of guinea pigs, each consisting of concurrently observed cortisone-treated and control animals. The first group was Schick tested with 0.02 M.L.D. of diphtheria toxin in 0.1 cc of normal saline in the plucked skin of the anterior abdominal wall. The second group was given lethal or near lethal doses of diphtheria toxin, some animals receiving 14 M.L.D., others 0.5 M.L.D. The last group of animals was injected with 14 M.L.D. diphtheria toxin and 10 units of diphtheria antitoxin at separate sites. Part of this group received antitoxin within one minute of toxin; the others were given antitoxin 4 hours after toxin injection. Cortisone acetate was administered in single daily doses of 5 mg for 2 days prior to and 4 days after injection of diphtheria toxin. Autopsies were performed on all animals. Hematoxylin and eosin sections of skin, heart, liver, spleen, and adrenals were taken in alternate animals.

Results. I. *Effect of cortisone on the Schick test.* The data summarized in Table I show that the time of appearance and the extent of skin lesions resulting from Schick testing were not significantly different in cortisone-treated and control animals. Sloughs and eschars formed at the sites of toxin injection in both and healed in approximately equal time.

II. *Effect of cortisone on guinea pigs given diphtheria toxin.* A. *14 M.L.D.* Large doses of diphtheria toxin were first used. Two control animals and 6 treated with cortisone died within 36 hours. At autopsy the outstanding findings were petechial hemorrhages in the viscera, with marked hemorrhages in the zona fasciculata of the adrenals. These findings were similar in both cortisone treated and control animals.

B. *0.5 M.L.D.*[†] When it became evident that cortisone was ineffective against a lethal dose of diphtheria toxin, it was decided to ascertain whether cortisone offered any protection against a near lethal dose, 0.5 M.L.D. The results are summarized in Table II.

From Table II it is evident that cortisone did not prevent death or influence survival time and weight loss in pigs given 0.5 M.L.D. diphtheria toxin. Skin lesions at the site of toxin injection developed rarely and were of equal extent in the 2 groups. Autopsies again showed petechial hemorrhages in visceral organs, with either gross or microscopic hemorrhages in the adrenals, particularly marked in the zona fasciculata. Cortisone did not influence the inflammatory response or the amount of hemorrhage in the sections studied (Fig. 1).

C. *Effect of mixing cortisone and diphtheria toxin in vitro prior to injection into guinea pigs.* In 3 animals 0.5 M.L.D. diphtheria toxin was mixed with 5 mg cortisone in the same syringe prior to injection to determine whether cortisone had any neutralizing

---

[†] 0.5 M.L.D. according to manufacturer's standardization.

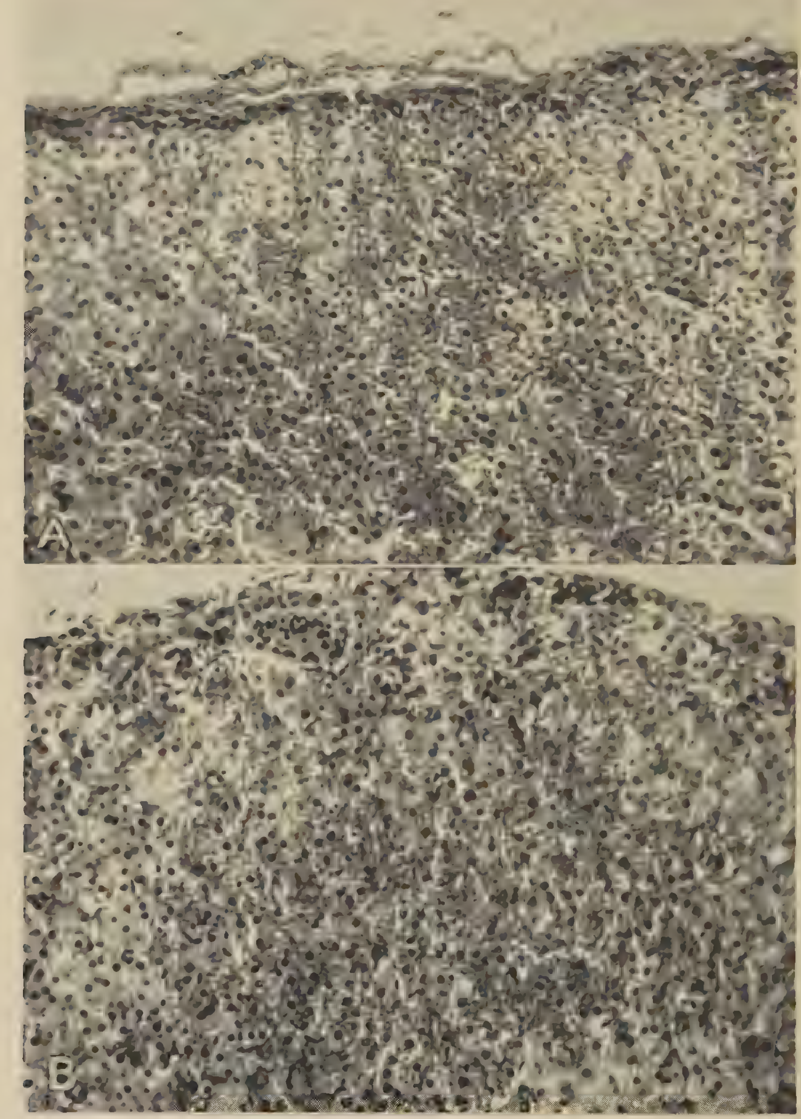


FIG. 1A. Adrenal gland of control guinea pig injected with 0.5 M.L.D. diphtheria toxin. Note hemorrhage and disruption of cord structure in zona fasciculata.

FIG. 1B. Adrenal gland of guinea pig injected with 0.5 M.L.D. diphtheria toxin and treated with cortisone. Note similarity to A.

effect, *in vitro,* on diphtheria toxin. All 3 animals died within 5 days of injection. Autopsy findings were similar to those in which toxin and cortisone were not mixed.

III. *Effect of cortisone on protective action of diphtheria antitoxin (10 units) against diphtheria toxin (14 M.L.D.).*

A. *Antitoxin injected simultaneously with toxin.* Diphtheria toxin and antitoxin were injected at separate sites within one minute of each other. Cortisone did not interfere with the life-saving property of diphtheria antitoxin. Sloughs and eschars formed at the site of toxin administration. Although these skin reactions appeared to be more extensive in cortisone-treated animals, the differences are not statistically significant (P lies between 0.1 and 0.2). Healing of skin lesions was incom-

TABLE III. Effect of Cortisone on Protective Action of Diphtheria Antitoxin (10 Units) Injected Simultaneously with Diphtheria Toxin (14 M.L.D.).

	No. of animals	No. of deaths	Time of death (days)	Mean and stand. dev. of max area of skin reaction (cm^2)
Controls	12	3	4, 7, 7	7.4 ± 5.8
Cortisone-treated	12	2	1, 4	12.5 ± 10.6

plete at the end of 3 weeks, at which time the animals were sacrificed. The results are summarized in Table III.

B. *Antitoxin injected 4 hours after toxin.* The previous experiment established that the protective action of diphtheria antitoxin, when administered within one minute of diphtheria toxin, was not adversely affected by cortisone. This experiment was designed to determine whether the fatal effect of a 4-hour delay in antitoxin administration(6) would be prevented by cortisone. Such a situation would simulate the delay in administration of antitoxin which usually obtains in human diphtheria infections.

Six controls and 6 cortisone-treated pigs all died within 36 hours of toxin injection. Skin lesions at the site of toxin administration did not develop in either group. Autopsy findings were similar in both cortisone-treated and control pigs.

Discussion. From this study it is apparent that cortisone in the dosage used did not significantly influence the effects of diphtheria toxin in guinea pigs. Tissue destruction, adrenal hemorrhages, and reactive inflammation were similar in cortisone-treated and control animals. In retrospect, if diphtheria toxin acts by interfering with internal oxidation in host cells, as suggested by Pappenheimer and Williams(7), one might not have expected cortisone to mitigate diphtheria intoxication. In accord with the results of the present study, Appel *et al.*(8) showed that Schick and Dick tests in humans were not affected by cortisone or ACTH. Spector and Mercer(9) concluded that ACTH did not prevent death in mice given tetanus toxin. The healing time of skin lesions produced by diphtheria toxin was not significantly altered by cortisone. In a study of the gross appearance and healing time of operative incisions in humans, Thompson(10) concluded that cortisone, in doses of 50 mg daily for one week, produced no noticeable effect. Recently, in spite of continued treatment with cortisone, healing of a traumatic fracture occurred normally on 2 occasions in a girl with disseminated lupus erythematosus, studied by the authors. This child had developed a marked Cushing's syndrome as a result of 23 months of cortisone therapy. There have been several reports, however, suggesting that cortisone does partially suppress fibroblastic activity(11-13).

Whereas diphtheria toxicity was not suppressed by cortisone, it seems important to emphasize that the protective action of diphtheria antitoxin, when injected soon after toxin, was not adversely affected by cortisone. These experiments strongly suggest, therefore, that antigen-antibody reaction, as exemplified by diphtheria toxin and antitoxin, is not influenced by cortisone.

Summary. 1. Cortisone did not affect the development of positive Schick reactions in non-immunized guinea pigs. 2. Cortisone did not prevent death, significantly increase survival time or prevent adrenal hemorrhages in guinea pigs given diphtheria toxin. 3. Cortisone did not interfere with the protective action of diphtheria antitoxin in guinea pigs given diphtheria toxin. 4. Cortisone had no direct neutralizing effect on diphtheria toxin *in vitro*.

---

1. Kligman, A. M., Baldridge, G. D., Rebell, G., and Pillsbury, D. M., *J. Lab. Clin. Med.*, 1951, v37, 651.

2. Kass, E. H., Ingbar, S. H., Lundgren, M. M., and Finland, M., *J. Lab. Clin. Med.*, 1951, v37, 780.

3. Mogabgab, W. J., and Thomas, J., *J. Lab. Clin. Med.*, 1950, v36, 968.

4. Spain, D. W., and Molomut, N., *Am. Rev. Tub.*, 1950, v62, 337.

5. Cluxton, H. E., Jr., 2nd Clin. ACTH Conf. (Mote), 1950, v2, 445.

6. Zinsser, H., Enders, J. F., and Fothergill, L. D.,

Imm. Prin. and App. in Med. and Pub. Health, 1939, Ed. 5, p. 504.

7. Pappenheimer, A. J., Jr., and Williams, C. M., *J. Gen. Physiol.,* 1952, v35, 727.
8. Appel, S. B., Fulton, L. A., and Orr, K. D., *U. S. Armed Forces Med. J.,* 1952, v3, 373.
9. Spector, S., and Mercer, R. D., *J. Inf. Dis,* 1951, v89, 224.
10. Thompson, I. M., personal communication, unpub. data.
11. Zoger, S., *Yale J. Biol. and Med.,* 1952, v25, 202.
12. Dougherty, T. F., and Schneebeli, G. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 854.
13. Ragan, C., Howes, E. L., Plotz, C. M., Meyer, K., and Blunt, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 718.

Received June 4, 1953. P.S.E.B.M., 1953, v83.

Intrinsic Factor Studies I. Paper Electrophoresis of Mixture of Gastric Juice and Radioactive Vitamin B_{12}.* (20398)

ROBERT F. SCHILLING AND WILLIAM P. DEISS.†

From the Department of Medicine, University of Wisconsin Medical School.

The simultaneous oral administration of normal human gastric juice and small amounts of vit. B_{12} to patients with pernicious anemia in relapse results in prompt hematopoiesis, while a similar amount of B_{12} without gastric juice is ineffective(1). The parenteral injection of microgram quantities of vit. B_{12} causes an excellent hematopoietic response in such patients(2). The simplest explanation of these observations is that the intrinsic factor activity of gastric juice promotes the intestinal absorption of the vitamin, either by means of a chemical binding of the B_{12} or by acting on the intestinal mucosa in such a manner as to facilitate the transport of the vitamin across the mucosa(3). Binding of vit. B_{12} has been studied most extensively by microbiological assay, and it has been shown that gastric juice combines with the vitamin in such a way as to render it unavailable to certain microbes(4). Several other substances, some known not to be active as intrinsic factor, combine with vit. B_{12} in a similar fashion (5,6).

This paper describes experiments designed to localize by paper electrophoresis the vit. B_{12} binding fraction in human gastric juice using radioactive vit. B_{12} (B_{12} Co^{60})‡(7) as a tracer.

Methods. a) Preparation of gastric juice. Gastric juice secreted after histamine injection was collected by a Levine tube from fasting normal subjects or patients with a peptic ulcer or an anxiety state. The juice in the stomach before histamine stimulation was not used. Only specimens showing an acid reaction to Toepfer's reagent were processed. After filtration through gauze to remove some of the mucus the juice was titrated with sodium hydroxide until neutral to brom thymol blue. After suction filtration through coarse sintered glass, the juice was stored at 4° or —10°C. Concentration of gastric juice was effected by dialysis against tap water and freeze-drying. In some experiments B_{12} Co^{60} was added before freeze-drying, and in others the powdered gastric juice was dissolved in an aqueous solution of the vitamin before application to the paper strip. b) *Paper electrophoresis.* A technic previously described(8) was used in these studies. Schleicher and Schuell No. 598 paper was found satisfactory for this work. Fifty microliters of the concentrated gastric juice-B_{12} Co^{60} mixture was applied to the paper, and after electrophoresis

---

* This investigation was supported by a research grant from the National Institutes of Health, U. S. Public Health Service.

† Research Fellow of the Arthritis and Rheumatism Foundation.

‡ Supplied through the generosity of Charles Rosenblum of Merck & Co., Rahway, N. J. The specific activity of the vitamin was approximately 64 microcuries/mg.

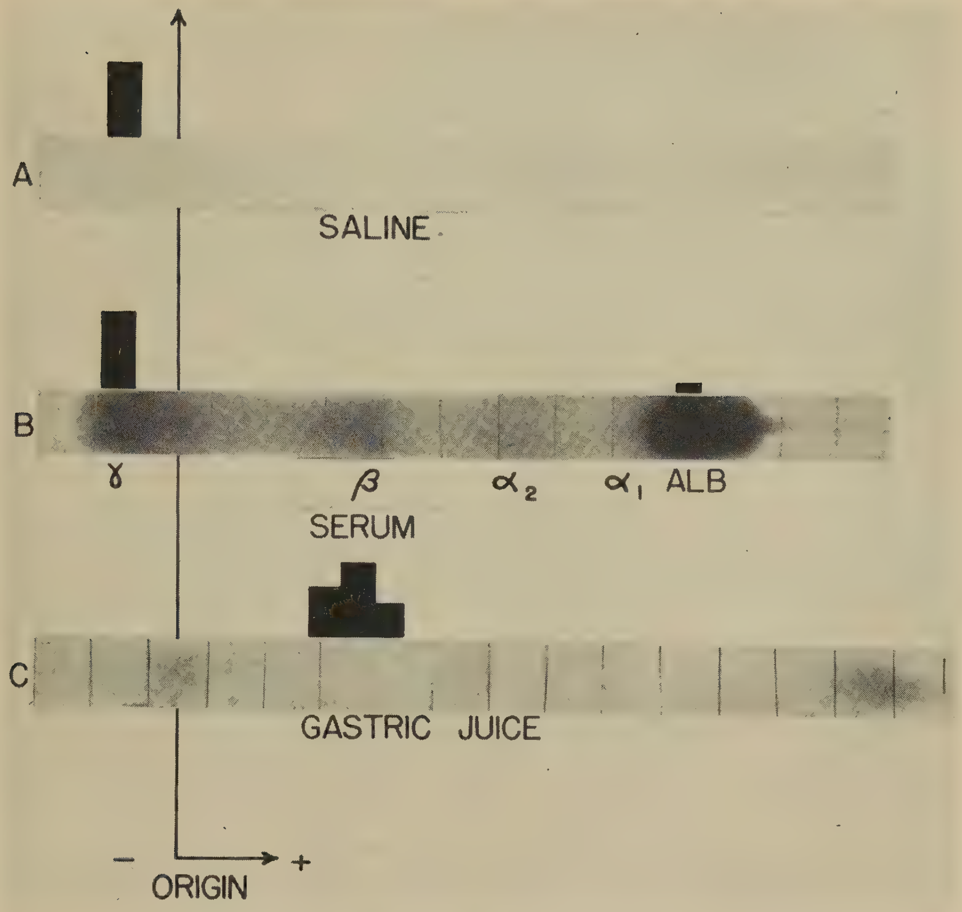


FIG. 1. Localization of radioactivity after simultaneous electrophoresis of B_{12} Co^{60} mixed with 0.9% NaCl (A), serum (B), and gastric juice (C). The bar graphs above the photographs of the stained paper strips represent schematically the localization of radioactivity.

in 0.075 M veronal buffer, pH 8.6, at 4°C for about 16 hours at 1.5 milliamperes, the paper was dried, cut transversely into 17 segments each 2 cm long, and the radioactivity of each segment determined in a windowless gas flow Geiger counter. These segments were subsequently stained with brom phenol blue to localize the protein spots.

Results. Fig. 1 compares the localization after simultaneous electrophoresis of B_{12} Co^{60} mixed with saline (a), with serum (b), and with gastric juice (c). It will be noted that the radioactive material has moved toward the cathode when mixed with saline or serum. There is also a very small amount of radioactivity associated with the albumin of the serum, evidence that this fraction bound some B_{12} Co^{60}. (This sample of vit. B_{12} contained no inorganic Co^{60}.) In a second experiment, a smaller quantity of B_{12} Co^{60} of higher specific activity was added to serum before electrophoresis, and the majority of the radioactivity was associated with the albumin. Since movement of B_{12} Co^{60} toward the cathode was noted in saline as well as in serum it would seem likely that this movement could best be attributed to electro-osmosis of "unbound" B_{12} Co^{60} in these experiments. The apparent association of radioactivity with the gamma globulin fraction of serum, therefore, is considered fortuitous and does not necessarily represent binding by this fraction.

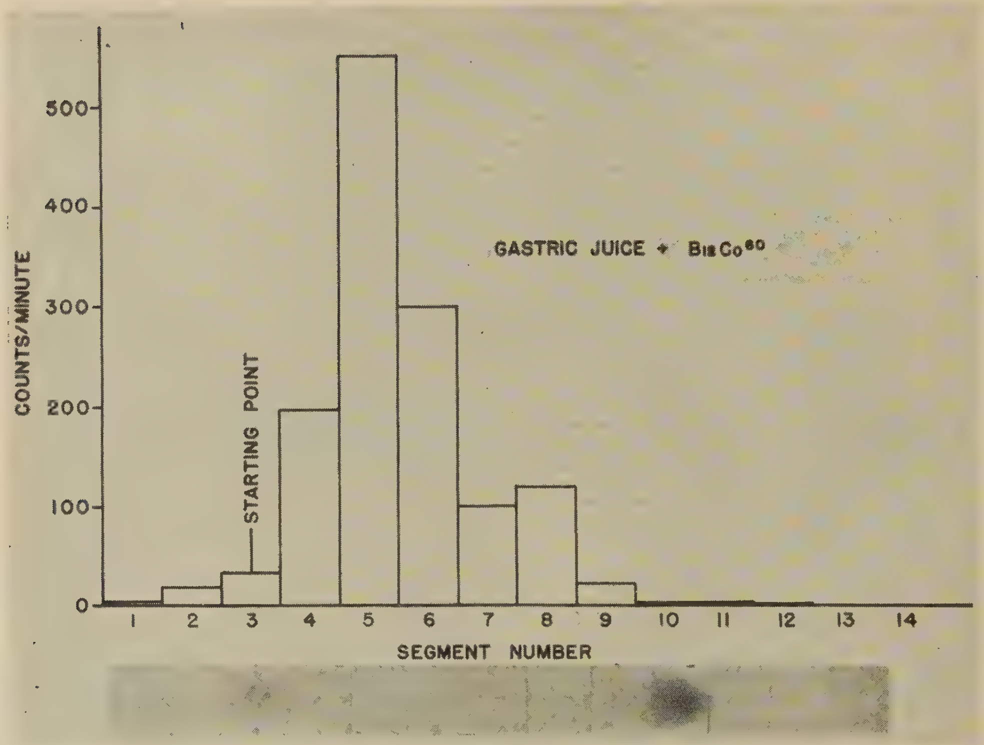


FIG. 2. Localization and quantitation of radioactivity after paper electrophoresis of a mixture of gastric juice and B_{12} Co^{60}. Current was applied for a shorter time here than in experiment pictured in Fig. 1.

When the B_{12} Co^{60} was mixed with gastric juice, it was bound to some component moving toward the anode. This behavior was noted in all eight of the experiments performed using 6 different gastric juices. That this component was not one of the 2 major protein peaks is evident from Fig. 1C and Fig. 2. One of the protein peaks remained at the starting point, and the other moved faster than the serum albumin. The B_{12} Co^{60} was not bound to either of the two detected protein peaks in gastric juice.

The effect of boiling the mixture of gastric juice and B_{12} Co^{60} on this binding is demonstrated in Fig. 3. It will be noted that, whereas definite localization of the radioactivity occurs when the vitamin and unboiled gastric juice are subjected to electrophoresis, the radioactivity is not well localized when the mixture was boiled.

Discussion. The data reported herein demonstrate that gastric juice contains a component, distinct from the major protein components, capable of binding vit. B_{12}. These observations of themselves are no evidence that this vit. B_{12} binding fraction is intrinsic factor. Using paper electrophoresis under different conditions of technic and buffer than those utilized in our experiments, Latner and Ungley(9) found at least two fractions of gastric juice which acted as intrinsic factor and were capable of binding vit. B_{12} as determined by microbiological assay. Differences in technic may explain the discrepancy between results reported here and those of Latner and Ungley *et al.*(9).

Summary. 1. Results of paper electrophoresis of mixtures of radioactive vit. B_{12} and concentrated human gastric juice demonstrate that the radioactive B_{12} is bound to a component of gastric juice which is not one of the major protein peaks and moves toward the anode. This behavior of the vit. B_{12} was not noted when it was mixed with saline or serum. 2. This localization of vit. B_{12} to a specific portion of gastric juice by paper electrophoresis may be a clue to the further identification of the intrinsic factor of Castle.

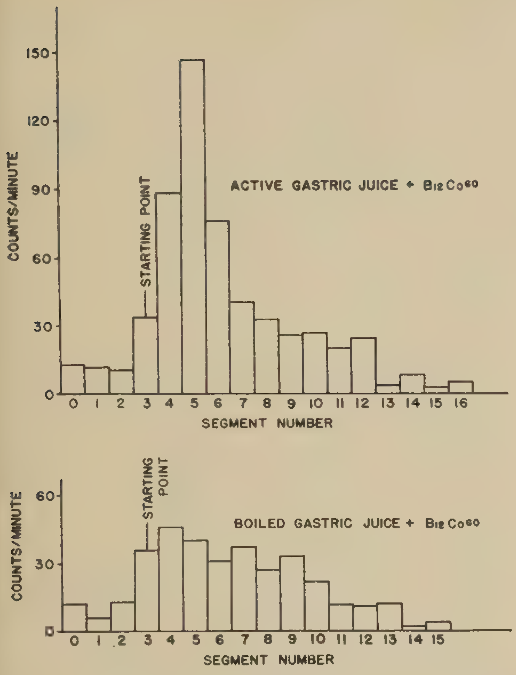


FIG. 3. Comparison of localization of radioactivity after simultaneous paper electrophoresis of boiled and unboiled aliquots of a mixture of gastric juice and B_{12} Co^{60}.

---

1. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R., and Epstein, M., *New Eng. J. Med.*, 1948, v239, 911.

2. Schilling, R. F., Harris, J. W., and Castle, W. B., *Blood*, 1951, v6, 228.

3. Wallerstein, R. O., Harris, J. W., Schilling, R. F., Castle, W. B., *J. Lab. and Clin. Med.*, 1953, v41, 363.

4. Ternberg, J. L., and Eakin, R. E., *J. Am. Chem. Soc.*, 1949, v71, 3858.

5. Beerstecher, E., and Altgelt, S., *J. Biol. Chem.*, 1951, v189, 31.

6. Bird, O. D., and Hoevet, B., *J. Biol. Chem.*, 1951, v190, 181.

7. Chaiet, L., Rosenblum, C., Woodbury, D., *Science*, 1950, v111, 601.

8. Deiss, W. P., Albright, E. C., and Larson, F. C., *J. Clin. Invest.*, 1952, v31, 1000.

9. Latner, A. L., Ungley, C. C., Cox, E. V., McEvoy-Bewe, E., and Raine, L., *Br. Med. J.*, 1953, v1, 4808.

Received June 4, 1953. P.S.E.B.M., 1953, v83.

Growth of Deciduomata in Adrenalectomized Rats. (20399)

JOSEPH T. VELARDO, FREDERICK L. HISAW,* AND ALDEN B. DAWSON.†
From the Biological Laboratories, Harvard University, Cambridge, Mass.

Recent reports by Pomeau-Delille(1), Swingle *et al.*(2), and Hisaw *et al.*(3) have clearly shown that decidual development can proceed in bilaterally adrenalectomized rats. The paper by Hisaw *et al.*(3) further indicated that the antimesometrial and mesometrial decidual tissue were histologically comparable in intact and adrenalectomized rats, and that the metrial glands were indistinguishable in the two groups as well. The present study is chiefly concerned with a series of experiments devised to ascertain the difference in the weights of uteri containing decidual tissue from the fifth full day after uterine traumatization to the twenty-first day of prolonged pseudopregnancy.

Procedures. Estrous rats were bilaterally adrenalectomized 12 hours after cervical stimulation(4) and were then divided into 2 groups. One was given 1% NaCl drinking solution and the other tap water. Vaginal smears were taken daily, fixed in methyl alcohol, and were then stained in Giemsa stain. Five full days after electrical stimulation of the uterine cervices, both uterine horns were traumatized throughout their entire length by scratching the antimesometrial surface with a burred needle. Laparotomies were performed 72 hours after uterine traumatization to es-

---

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

† Aided by a grant from the William F. Milton Fund, Harvard University.

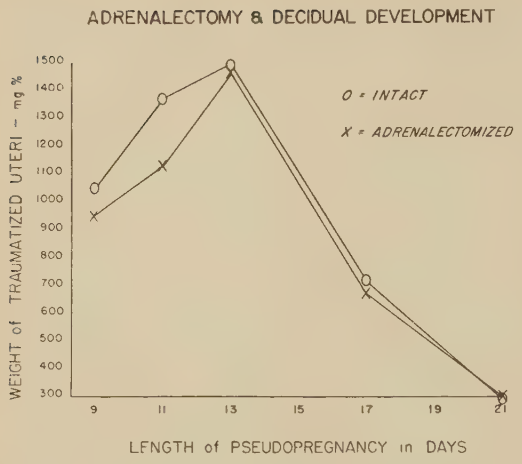


FIG. 1. A graph showing the similarity in the weights of uteri containing decidual tissue in intact and adrenalectomized rats.

tablish the presence of deciduomata. Groups containing 7 to 10 animals were killed on the 9th, 11th, 13th, 17th, and 21st days of pseudopregnancy (*i.e.*, 5, 7, 8, 13 and 17 full days after the uteri were traumatized). The uteri were removed and weighed to the nearest tenth of a milligram on a Roller-Smith torsion balance. The same procedures were used in the non-adrenalectomized control series.

Results and discussion. Developing deciduomata were found in both the adrenalectomized (with and without 1% NaCl drinking solution) and intact control rats when laparotomized 72 hours after uterine traumatization. Forty-eight hours later, on the 9th day of pseudopregnancy, the first 2 groups of rats were killed, and the uteri were removed and weighed. Examination of the arithmetical means plotted in Fig. 1 clearly shows that there is only a slight difference in the weight of the uteri in both groups at this time. There is a somewhat larger difference in the weight responses on the 11th day of pseudopregnancy, but the gross morphology of the deciduomata in both groups was essentially the same. That adrenalectomy exerts no appreciable effect on the growth of the decidual reaction is further borne out in the data obtained on the 13th day of pseudopregnancy (Fig. 1) which show that the weights of the uteri are exactly the same. It is of further interest to note here that the morphology of the deciduomata is indistinguishable in the 2 experimental groups as well(3,5). Also, the involutionary process of the decidual reaction in both adrenalectomized and non-adrenalectomized rats indicates additional similarity. In brief, it is readily apparent that the absence of the adrenal glands does not modify the rate at which deciduomata undergo atrophy and total dissolution (Fig. 1). Thus, these data, obtained from gravimetric and histologic studies, support the idea that bilateral adrenalectomy does not modify the morphology and weight of the decidual reaction throughout its entire development and ensuing atrophy in the rat.

Our present knowledge of the physiologic activity of the adrenal glands during pregnancy, pseudopregnancy, and prolonged pseudopregnancy is so limited that it is not possible to speculate on the nature of the role of the adrenal glands during these physiologic states. Since it has been demonstrated that injections of adrenal cortical steroids and adrenocorticotropic hormone can inhibit decidual development and cause pregnant rabbits, rats, and mice to abort(6-8), it seems probable that in the normal animal a balanced relationship is maintained between these steroids and those responsible for the maintenance of pregnancy. That something of this nature might be so is indicated by recent reports showing that (a) placental extracts will decrease the size of the adrenal glands(9), (b) the weight of the adrenal glands decreases during the growth of deciduomata(10), and (c) adrenal cortex-like hormones have been isolated from human and equine placental tissue(11).

Summary and conclusions. Both histologic and gravimetric data show conclusively that the growth and subsequent regression of deciduomata in bilaterally adrenalectomized pseudopregnant rats are comparable in every respect to those formed in intact rats. Substitution therapy is not required when the adrenal glands are removed 12 hours after electrical stimulation of the uterine cervix.

---

1. Pomeau-Delille, G., *Compt. Rend. Soc. Biol.*, 1949, v143, 1251.

2. Swingle, W. W., Fedor, E. J., Barlow, G. Jr., Collins, E. J., and Perlmut, J., *Am. J. Physiol.*,

1951, v167, 593.

3. Hisaw, F. L., Velardo, J. T., and Dawson, A. B., *Anat. Rec.*, 1952, v113, 87.

4. Greep, R. O., and Hisaw, F. L., PROC. SOC. EXP. BIOL. AND MED., 1938, v39, 359.

5. Velardo, J. T., Dawson, A. B., Olsen, A. G., and Hisaw, F. L., *Anat. Rec.*, 1952, v112, 98.

6. Hisaw, F. L., and Velardo, J. T., *Endocrinology*, 1951, v49, 732.

7. Robson, J. M., and Sharaf, A. A., *J. Physiol.*, 1951, v114, 11p.

8. ———, *J. Physiol.*, 1952, v116, 236.

9. Vilardell, R., *Gynecol. et Obstet.*, 1929, v20, 65.

10. Velardo, J. T., and Hisaw, F. L., Unpublished data.

11. Johnson, R. H., and Haines, W. J., *Science*, 1952, v116, 456.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Effect of Thyroid Hormone on Growth of Thyrotrophin-Secreting Pituitary Tumors.* (20400)

EVELYN L. GADSDEN, AND JACOB FURTH.

From Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Tumors of the pituitary develop in almost every mouse following destruction of the thyroid by I^{131}(1,2). These tumors secrete large quantities of thyroid-stimulating hormone (TSH)(3). The mechanism of the development of these tumors is not known with certainty. Lack of TH (thyroid hormone) provides the major stimulus for proliferation of TSH secreting cells, but whether or not ionizing radiation, incidental to radiothyroidectomy, is an essential contributing factor is under investigation(4,5). These induced tumors are readily transplantable on radiothyroidectomized but not on normal hosts(2). However, in the course of subpassages the tumors may gain autonomy and grow even better in normal than in radiothyroidectomized animals(3). Induction of the primary tumors can be prevented by administration of TH(6,7). If lack of TH is the stimulus for the growth of these tumor cells, can the growth of tumor grafts be prevented by TH? Will TH also affect the growth of autonomous tumors? Will thiouracil, an antithyroid compound which blocks the synthesis of TH, promote the growth of dependent tumors? The 3 sets of experiments now to be reported were designed to answer these questions.

Materials and methods. C57 black mice of both sexes were used. Transplantations of the I^{131}-induced pituitary tumors were made by cutting the tumor into fine particles in physiological saline and injecting it into the thigh muscle. Thyroid hormone was administered (a) by feeding desiccated thyroid in food or (b) by intramuscular injections or subcutaneous grafts of enlarged thyroids obtained from mice bearing autonomous grafted pituitary tumors. In one experiment (Table I) thyroids of tumor-bearing mice fed thiouracil for a long period of time were used. The desiccated thyroid† was mixed with ground Purina Chow, ½ g thyroid per 1000 g food. Each animal was fed approximately 3 g of this food mixture per day (1½ mg thyroid). Thyroids to be grafted were either cut into fine particles in physiological saline and injected in the muscle of the thigh or were grafted subcutaneously. Thyroid feeding was usually begun on the day of tumor grafting; the time relation is shown in the tables. Thiouracil‡ was administered as a 0.1% solution in drinking water given *ad libitum*. Thiouracil treatment was begun prior to tumor grafting in most experiments and at the time of tumor grafting in 2 experiments as indicated in Table III.

Results. Effect of TH on dependent tumors.

---

* Work performed under contract for the U. S. Atomic Energy Commission.

† "Thyroid Strong" obtained through the courtesy of Parke-Davis.

‡ Obtained through the courtesy of the American Cyanamid Co.

TABLE I. Effect of Thyroid Hormone on Growth of Dependent Pituitary Tumors.

			Radiothyroidectomized hosts				Normal hosts	
	TH given		TH treated		Not treated		Not treated	
Tumor strain	Time, days*	Mode	No. inj. / No. takes	Latency, days†	No. inj. / No. takes	Latency, days	No. inj. / No. takes	Latency, days
Strains remained dependent:								
101	0	Food	7/0	—	10/10	137 (124–158)	4/0	—
101	—3	Graft	7/0	—	10/10	134 (123–167)	10/0	—
124	+1	Food	10/0	—	7/7	78 (63–99)	11/0	—
124	0	Graft	7/2	137 (132–141)	8/8	116 (109–121)	10/0	—
77	—4	”	10/0	—	6/6	181 (143–205)	6/0	—
Strains turned autonomous:								
3-D	+4	Food	3/3	157 (148–161)	9/9	75 (65–90)	11/11	99 (83–133)
3-D	0	Graft‡	5/5	83 (75–96)	4/4	59 (54–75)	5/5	211 (159–236)
163	0	”	2/0	—	9/9	81 (56–125)	9/2	139 (139)
19	—8	”	8/6	132 (68–208)	7/7	80 (68–110)	8/0	—

* In relation to pituitary tumor graft.
† Means; range is in parentheses.
‡ Hyperplastic thyroid was obtained from thiouracil-treated mice.

TABLE II. Effect of Thyroid Hormone on Growth of Autonomous Pituitary Tumors.

		Radiothyroidectomized hosts				Normal hosts			
TH given		TH treated		Not treated		TH treated		Not treated	
Time, days*	Mode	No. inj. / No. takes	Latency, days†	No. inj. / No. takes	Latency, days	No. inj. / No. takes	Latency, days	No. inj. / No. takes	Latency, days
0	Food	3/3	76 (66–96)	3/3	117 (117)	5/4	66 (66)	5/5	47 (40–54)
0	”	5/5	46 (29–81)	5/5	46 (40–60)	10/10	39 (29–40)	11/11	27 (16–29)
+1	”	—	—	10/10	33 (21–51)	10/10	19 (16–21)	10/10	13 (11–16)
0	Graft	—	—	5/5	111 (74–177)	4/4	49 (42–63)	7/7	53 (42–74)
0	”	5/5	30 (30)	5/5	32 (30–40)	5/5	47 (47)	5/5	30 (30)
—6	”	3/3	30 (30)	7/6	75 (70–80)	3/3	30 (30)	10/10	32 (30–58)
0	”	—	—	10/10	77 (55–118)	5/5	54 (46–90)	5/5	46 (46)
–13	”	3/3	84 (70–97)	4/4	96 (86–107)	—	—	6/6	58 (53–70)

*† For explanations, see Table I.

The 9 experiments can be presented in 2 groups: (a) In 5 experiments 3 transplantable tumor strains were used which thus far did not turn autonomous. Grafts of these failed to grow in the 41 normal mice used in these studies, but grew in all but one of the 40 radiothyroidectomized mice. Thirty-nine radiothyroidectomized mice grafted with such tumors were also treated with TH and of these only 2 developed tumors. Thus TH was effective in preventing tumor growth. In the 2 animals in which the tumors grew the thyroid grafts could not be identified at autopsy; thus the tumor growth could be explained by unsuccessful thyroid graftings. (b) In 4 experiments strains of pituitary tumors were used which had a tendency to become autonomous. Eighteen of 33 normal mice were receptive to grafted tumors of these strains which appeared after a long latency period. The tumors grew after a shorter latency period in all of 29 grafted radiothyroidectomized mice. The administration of TH to radiothyroidectomized mice reduced the number of successful tumor grafts and increased their latency period (Table I).

Inhibitory effect of TH on growth of established pituitary tumors. In experiments performed on a small scale TH retarded the growth of already established tumors but did not cause their complete regression.

Two preparations containing TH were used. (a) thyroxine (Roche-Organon) was given 6 times weekly in doses of 0.5 μg in 0.05 cc of saline and (b) thyroprotein (Parke-Davis) was given 6 times weekly in doses of 0.125 mg in 0.1 ml of distilled water.

In one experiment thyroxine was adminis-

TABLE III. Effect of Thiouracil on Growth of Pituitary Tumors.

Tumor strain	Thiouracil treatment, days	Hosts					
		Radiothyroidectomized		Thiouracil-treated		Normal	
		No. inj. / No. takes	Latency, days	No. inj. / No. takes	Latency, days	No. inj. / No. takes	Latency, days
Strains remained dependent:							
82	—16	5/5	125 (105–161)	5/0	—	4/0	—
101	—126	9/9	134 (121–157)	4/0	—	10/0	—
Strains turned autonomous:							
3-D	0	8/8	75 (61–103)	5/5	104 (94–136)	—	—
3-D	0	6/6	73 (65–75)	4/4	233 (197–267)	5/5	246 (230–267)
3-D	0	6/6	85 (74–100)	5/5	174 (165–188)	4/4	226 (215–236)
3-D	—93	8/8	67 (53–90)	6/6	175 (165–186)	8/8	222 (207–251)
3-D	—24	5/5	56 (42–60)	6/6	101 (84–137)	6/6	173 (163–178)
3-D	—89	5/5	70 (62–85)	8/8	79 (72–85)	5/4	113 (113)
163	—117	9/9	39 (32–59)	9/9	121 (107–172)	10/7	268 (210–282)
3A	—127	4/4	84 (71–95)	4/4	59 (46–71)	5/5	51 (46–56)

For explanations, see Table I.

tered for 8 weeks to 4 animals each having tumors ranging from 1 to 3 cm across. Control animals with tumors of comparable sizes were observed without treatment. In 3 of the treated animals the tumors regressed, though not completely; the size of the tumor of the fourth animal remained nearly unchanged. The control animals died with very large, progressively growing tumors.

In another experiment thyroprotein was administered to 2 mice for 4 months. The treatment began when the tumors measured about 0.5 x 2 cm. These tumors greatly diminished in size, measuring at death 5 x 8 mm. The tumor of a control mouse grew progressively, and the animal died with a large tumor.

Another 3 mice were injected with thyroprotein when their tumors measured about 2 cm across. Tumors in 2 of these mice failed to progress during 15 weeks of treatment. The 2 controls died with large tumors. In another experiment the tumors of 2 mice remained about unchanged in size after 14 weeks of thyroprotein injections and these animals lived much longer than the controls which died with large tumors.

Although the total number of animals in these experiments is small, the results are highly significant since several strains of tumors were used in these studies and progressive growth occurred invariably in hundreds of untreated mice with grafted tumors.

Effect of TH on autonomous tumors. In all 8 experiments listed in Table II the autonomous strain 3-A was used. These autonomous tumors grew in all but one of the 150 mice grafted, and it is possible that the animal in which the tumor failed to grow did not receive viable cells. Table II indicates that while TH treatment failed to prevent tumor growth in any of the animals, it hastened tumor growth somewhat in most radiothyroidectomized mice. In normal hosts the results were variable. In some experiments the latency period was somewhat longer; in others, somewhat shorter, indicating that the mild TH treatment did not significantly alter the growth rate of the tumors. The thyroids of mice bearing autonomous pituitary tumors were greatly enlarged and the amount of TH secreted by them was probably greater than that administered. What the continued administration of very large quantities of TH would do remains to be studied.

A correlation of the size of thyroid grafts with tumor growth was not made. When the grafts were identified and studied microscopically, they exhibited the same changes as those seen in the thyroids of the hosts, namely, hyperplasia, reduction of colloid content, intracellular colloid deposits and formation of adenomas.

Effect of thiouracil on the growth of pituitary tumors. The 10 experiments performed can be conveniently presented in 3 groups: (a) In 2 experiments in which tumor strains were used which remained dependent in the course of a large series of transplantation studies (not detailed in this report), the grafts succeeded in all of 14 radiothyroidectomized mice and in none of 14 normal mice. Thiouracil treatment did not render the 9 animals studied susceptible to these tumors. (b) In 7 experiments strains of pituitary tumors were used which tended to become autonomous. Transplantations succeeded in all of 47 radiothyroidectomized mice and failed in 4 of the 38 normal control mice. In the radiothyroidectomized animals the average latency period varied between 39 and 85 days; in normal mice between 113 and 268 days. The tumor grafts succeeded in all of 43 thiouracil treated mice. The latency period was in every experiment shorter than in normal hosts and much longer than in radiothyroidectomized hosts (Table III). (c) In one experiment an autonomous pituitary tumor strain was used. The grafts succeeded in all animals and appeared first in normal, next in thiouracil-treated, and last in radiothyroidectomized hosts. These experiments indicate that blocking TH synthesis by thiouracil does not accomplish the results obtained by massive destruction of the thyroid with radioiodine. In an earlier study the effect of thiouracil treatment on tumor growth was compared with that of depression of the thyroid by graded doses of radioiodine, and it was estimated that the thiouracil effect was equivalent to that of a small dose of I^{131} ($\sim 25\ \mu c$) which caused but slight depression of the thyroid.

Thiouracil-treated animals had greatly enlarged thyroids which were studded with adenomas, as described by others.

Summary and conclusions. 1. The administration of thyroid hormone effectively prevents the growth of dependent pituitary tumors induced in and grafted on radiothyroidectomized mice. Autonomous pituitary tumors grow faster in normal than in radiothyroidectomized hosts, and, in the latter, administration of TH hastens the growth of the tumor. Sustained thiouracil treatment does not render normal mice susceptible to dependent pituitary tumors but hastens somewhat the growth of such pituitary tumors which tend to become autonomous. 2. These experiments support the view that lack of TH is the major growth stimulant of pituitary growths in radiothyroidectomized mice and that sustained lack of this hormone is essential for the proliferation of these tumor cells. In the course of sustained proliferation a new (autonomous) type of tumor cell arises which is not only uninhibited but is often stimulated by TH, the physiological depressant of normal pituitary cells producing thyrotrophin.

---

1. Gorbman, A., *J. Clin. Endocrinology*, 1950, v10, 1177.
2. Furth, J., and Burnett, W. T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 222.
3. Furth, J., Gadsden, E. L., and Burnett, W. T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 4.
4. Gorbman, A., and Edelmann, A., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 348.
5. Furth, J., *Cancer Research*, 1953, in press.
6. Gorbman, A., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 538.
7. Goldberg, R. C., and Chaikoff, I. L., *Endocrinology*, 1951, v48, 1.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Human "Analgesic" Action of d-Amphetamine, Amobarbital, Acetylsalicylic Acid and Acetophenetidin, in Combination. (20401)

STANLEY C. HARRIS, AND ROBERT C. WORLEY.

From the Department of Physiology and Pharmacology, Northwestern University Dental School, Chicago, Ill.

This new combination of drugs to be considered as analgesic evolved from 2 sets of pharmacological information. First, a mixture of amphetamine, aspirin and phenacetin, known as "Edrisal", has enjoyed widespread use as an analgesic since it was assayed and found effective by Burrill, Goetzl, and Ivy(1). This work, in turn, developed from the recognition that sympathomimetic drugs were capable of producing analgesic action(2). Second, the mood improvement following therapy with amphetamine is well established. Its combination with barbituric acid derivatives for the relief of anxiety and depression apparently originated with the work of Davidoff and Goodstone(3).

This report relates the experience obtained when the pain threshold elevating abilities of the following were compared on each of 16 normal human volunteers. A. d-amphetamine sulfate, 5 mg; amobarbital, 32 mg; acetylsalicylic acid, 162 mg, and acetophenetidin, 162 mg, hereafter called "Daprisal".* B. acetylsalicylic acid, 162 mg, and acetophenetidin, 162 mg. C. placebo. D. one experiment in which nothing was taken by the subjects, hereafter called the "dry-run".

Methods. To establish the experimental pain thresholds and compare the effects of the 4 treatments upon them, the method and discipline of Harris and Blockus(4) was employed. The thresholds of electrical elicitation of pain in a tooth pulp of each subject, were determined in triplicate 3 times at 15-minute intervals before medication; then 8 times at 20-minute intervals after medication. The subjects were selected because they had at least one metal restoration, which did not extend to touch soft tissue, in a vital tooth. Before every threshold determination, the tooth to be tested was carefully dried with 95% ethanol and, if necessary, kept dry by surrounding it with cotton rolls. Subjects were always in the post-cibal state. The medication program was determined in advance so that each treatment was given to every subject in such a way that each treatment occurred first, second, third and fourth, 4 different times. Further, the sequence of medications was arranged in 4 different patterns. Four envelopes were labeled for each subject and filled according to the above program before the experiment began. In this fashion, neither operator nor subjects knew the identity of medication at any time prior to the completion of the whole experiment. The occasion of the dry-run was an exception, of course. It should be stated that the 3 different forms taken by mouth were prepared as tablets that were identical in appearance.

Results. All readings taken were recorded in 4 tables, one for each treatment. An analysis of variance was performed on each of these 4 sets of data and are summarized in Table I.

Table I reveals that there was no significant variation between thresholds observed at 11 intervals over 3 hours (trials) during the dry-run or when aspirin-phenacetin was administered. However, the above variance was statistically significant ($P < 0.01$) when placebo or Daprisal was taken. The variances between trials in these latter 2 cases were broken down for more detailed analysis in Table I and showed that the post-medication thresholds were significantly higher than the pre-medication thresholds after Daprisal and placebo therapy ($P < 0.01$). There also was significant variation ($P < 0.01$) between post-medication thresholds after Daprisal but not after placebo.

To facilitate comparison of the time course of threshold changes after medication it is desirable to have a common base line. To do

---

* Trademark, Smith, Kline and French Laboratories, Philadelphia, Pa.

TABLE I. Summary of Analyses of Variance of Raw Data by Medications.

Medications	Source of variance	Degrees of freedom	Mean square	"F" ratio
Aspirin-phenacetin	Subjects	15	1032069	806.4 *
	Trials	10	1908	1.49
	Subjects × trials	150	1279	
Placebo	Subjects	15	1045701	412.22*
	Trials	10	11933	4.70*
	Subjects × trials	150	2536	
	Trials 1-3	2	5206	2.05
	” 4-11	7	1032	.40
	” 1-3 vs. 4-11	1	101695	40.08*
Daprisal	Subjects	15	1200360	253.54*
	Trials	10	51744	10.92*
	Subjects × trials	150	4734	
	Trials 1-3	2	2685	.56
	” 4-11	7	29478	6.22*
	” 1-3 vs. 4-11	1	276248	58.34*
Dry-run	Subjects	15	1161477	859.14*
	Trials	10	1383	1.02
	Subjects × trials	150	1351	

* = $P<0.01$.

TABLE II. Average Post-Medication Deviations from Pre-Medication Pain Thresholds for Each Drug and Control, Expressed in Microamperes.

	Minutes							
Medications	20	40	60	80	100	120	140	160
Aspirin-phenacetin	0.24	1.40	2.10	3.20	2.80	3.38	2.41	4.43
Placebo	5.93	6.18	4.51	3.49	5.44	6.87	5.30	4.79
Daprisal	0.52	6.78	8.07	9.31	11.52	14.26	16.17	20.12
Dry-run	0.68	0.46	−0.56	0.12	1.37	0.66	0.74	0.44

this, the average pre-medication threshold was calculated for each subject each time he served, and each of his post-medication changes from his pre-medication average was recorded. This procedure gave 4 new tables with one reading for each post-medication interval for each subject. These data are summarized in Table II and graphically presented in Fig. 1. To determine the significance of the differences between treatment effects, an analysis of variance was performed simultaneously on the data of all 4 of these tables. This analysis is summarized in Table III.

It is readily seen that there was significance ($P <0.01$) to the difference between post-medication changes in thresholds attributable to treatments. Detailed analysis of treatment variance indicates that the changes in threshold following Daprisal are significantly greater than after the other 3 treatments and that there is no significant difference between the effects upon threshold of Aspirin and phenacetin, placebo, and a dry-run. The significance of variance between trials is attributed to the continually increasing elevation of the threshold after Daprisal which had not leveled off 160 minutes after administration.

A third analysis of variance was run on all of the original data simultaneously to evaluate the variation between the readings taken in triplicate. The "F" ratio for this variance was negligible (0.001).

Discussion. In this laboratory and elsewhere, experience with electrical excitation of the tooth pulp has consistently failed to demonstrate significant elevation of the pain threshold by non-narcotic drugs.

Daprisal, however, is the first non-narcotic ever tested in this laboratory which caused a statistically significant elevation of the threshold to experimentally induced pain in human subjects.

It is to be noted that the above conclusion is based on the conservative analysis compar-

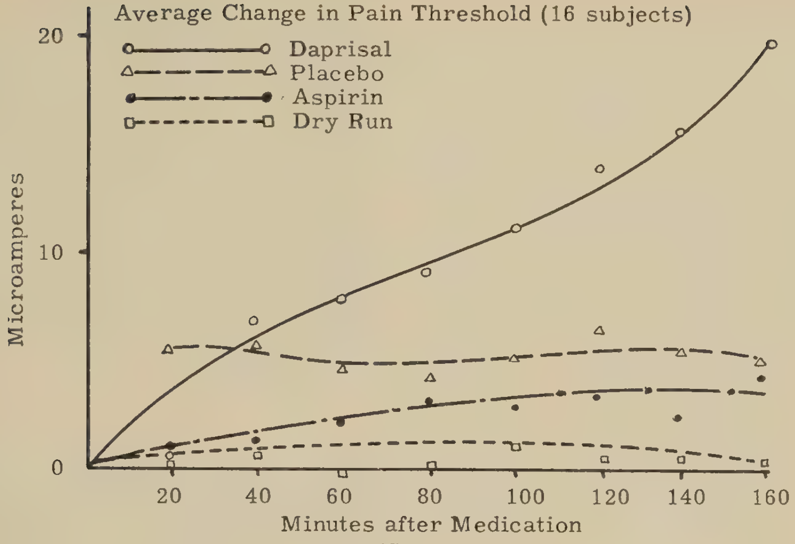


FIG. 1.

ing *all* post-medication readings rather than only maximal readings. Stated differently, the *average* threshold after Daprisal was significantly higher than were the *average* thresholds after aspirin-phenacetin, placebos, and no medication.

That the post-medication thresholds were higher than the pre-medication thresholds after placebos, is not readily explained and has not happened uniformly in our experience. Even in this respect, there was no significant variance between post-medication thresholds after placebos while the thresholds continued to rise significantly during the entire time after Daprisal had been given.

Worth mentioning was that not one of the 16 subjects noted any stimulation or depression after any of the drugs given. It was with some surprise that sleeplessness was not reported by anyone, although 5 mg of d-amphetamine was given, in combination, at 7:30 p.m. in every instance of its use.

Limited clinical experience in our hands tends to confirm the experimental finding that this unique drug combination is indeed analgesic. In several clinical cases, pain unrelieved by large quantities of salicylic acid derivatives was relieved by a single Daprisal tablet but not by a placebo. The mechanism by which compounds impotent as analgesics individually, become a potent analgesic when combined, is not sufficiently understood at this time to justify discussion.

Summary. Four treatments were compared on each of 16 human subjects for their ability to elevate the threshold to experimentally in-

TABLE III. Summary of Analysis of Variance of Post-Medication Deviations from Pre-Medication Averages by Subjects, Treatments and Consecutive Observations (Trials).

Source	Degrees of freedom	Mean square	"F" ratio
Treatments	3	440274.3	6.09*
Subjects	15	100054.1	1.38
Treatments × subjects	45	72245.2	
Trials	7	36461.4	5.94*
Error	441	6128.9	
Breakdown of treatments			
Daprisal vs. others	1	1065604.7	14.75*
Others	2	127609.1	1.76

* = $P < 0.01$.

duced pain of the tooth pulp, by the method and discipline of Harris and Blockus. Rigorous elimination of bias was exercised. The treatments were: A. d-amphetamine, 5 mg; amobarbital, 32 mg; acetylsalicylic acid, 162 mg; acetophenetidin, 162 mg; B. acetylsalicylic acid, 162 mg; acetophenetidin, 162 mg; C. placebo; D. no medication, *i.e.*, a dry-run. The threshold of experimentally induced pain was elevated more significantly during the 2½ hours after the amphetamine mixture than after the other 3 treatments.

1. Burrill, D. Y., Goetzl, F. R., and Ivy, A. C., *J. Dent. Res.*, 1944, v23, 337.
2. Ivy, A. C., Goetzl, F. R., Harris, S. C., and Burrill, D. Y., *Quart. Bull., Northwestern Univ. Med. Sch.*, 1944, v18, 298.
3. Davidoff, E., and Goodstone, G. L., *Psychiatric. Quart.*, 1942, v16, 541.
4. Harris, S. C., and Blockus, L. E., *J. Pharm. and Exp. Therap.*, 1952, v104, 135.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Demyelination in Lambs from Ewes which Feed on Seaweeds. (20402)

P. A. PÁLSSON AND H. GRÍMSSON. (Introduced by B. Sigurdsson)

From the Institute for Experimental Pathology, University of Iceland, Keldur, Reykjavik.

A nervous disorder in newborn and young lambs has been known to occur since the middle of the last century on many farms in Iceland *i.e.* farms near the coast where the sheep graze on seaweeds during the winter. Ewes are most prone to give birth to affected lambs when they eat much seaweed during the last half of the gestation period.

Many of the farmers along the coast graze their sheep on seaweeds in order to save hay. The daily intake of seaweeds per animal may become quite high (6 to 10 kg). Sheep seem to prefer certain species of seaweed such as *Rhodymenia palmata* and *Alaria esculenta* but where these species are scarce they consume other species too. The disease is rare on farms off the coast. Its incidence varies considerably from year to year on the same farm. On many farms the *mortality* may vary annually from 1% to 50% of the lambs born on the farm, but occasionally the losses may be as high as 80% to 90%. On some farms the disorder occurs every year, on other farms, even in the same area, the disorder seems to occur for one or 2 years after the herd has been free from the disorder for a number of years. Ewes which have given birth to affected lambs one year may produce either affected or healthy lambs the following year. This disorder is not so widespread now as it was 20-30 years ago, probably because the ewes are now fed much more hay during the gestation period, and on many farms the ewes are kept away from the seaside altogether during the last half of pregnancy. When the hay crop is poor for one year large losses among the lambs still occur. Older ewes seem to be more prone to give birth to affected lambs than young ewes, but ewes at any age may give birth to affected lambs.

The symptoms are usually essentially similar, but vary considerably in severity. The most prominent and common symptom is incoordination of movement, in many cases the lambs are unable to rise and stand, but lie helpless, some may be able to rise, but walk with much difficulty and collapse almost immediately, others walk with a staggering gait. Mild cases show only weakness of the hind quarters, especially when hustled. Many lambs are blind. The body temperature is normal. Most cases are fatal, but those lambs which do not show symptoms until some weeks after birth, often survive and if bred later, they may give birth to healthy lambs.

The macroscopic pathological lesions seem to be confined to the nervous system, particularly the cerebrum. In approximately one-half of the cases which we have examined, macroscopic lesions were present. In the most typical cases the convolutions of the cerebrum were flattened and poorly defined, on palpation fluctuation could easily be felt on the hemispheres and the cerebral cortex was in

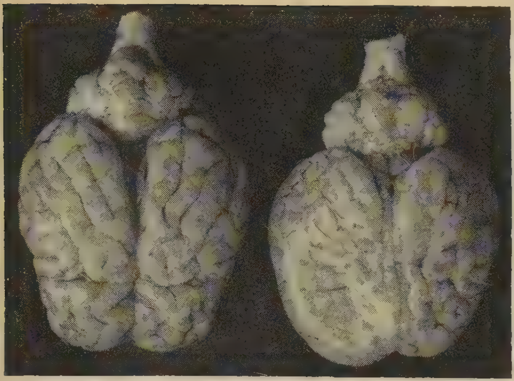


FIG. 1. Brain from a lamb which showed symptoms of ataxia (left). For comparison a brain from a normal lamb.

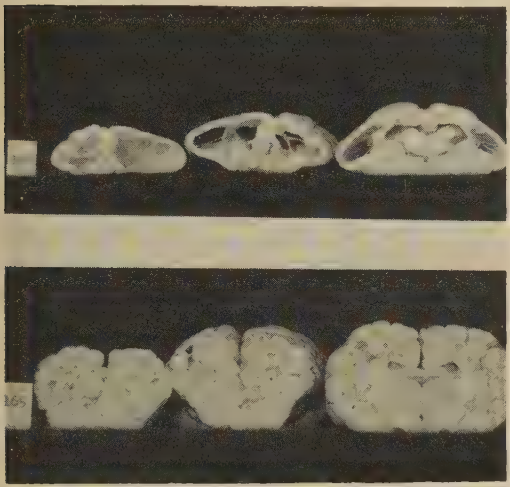


FIG. 2. Same brains as in Fig. 1. Series of coronal sections showing marked bilateral cavitation. Some dilatation of the lateral ventricles.

these cases extremely thin, (Fig. 1). When sliced into coronal sections a bilateral degeneration of the cerebral white matter was observed. The white matter had been almost completely liquified, and destroyed, leaving a cavity in which a network of a gelatinous substance was seen (Fig. 2). These cavities seemed to extend from the occipital pole and could in extreme cases be followed to the frontal pole. Around the cavitations the grey matter seemed to be fairly normal. In a few cases the lateral ventricles were somewhat distended. No hemorrhages were observed. The midbrain, cerebellum and the brain stem showed no macroscopic lesions and the meninges were normal. In the milder or delayed cases macroscopic lesions were usually absent. By comparing microscopic sections from affected lambs stained for myelin (Weigert-Pal) with corresponding sections from normal lambs stained in the same way, demyelination was found to be the characteristic lesion. In cases where macroscopic lesions had been prominent there seemed to be an almost total absence of myelin in the hemispheres. In other cases the destruction of cerebral myelin was less marked and in many cases only small foci of demyelination were observed. Focal or diffuse infiltration by inflammatory cells were not observed and hemorrhages were not seen.

As this condition resembles in many respects "swayback" as described by Innes and Shearer(1) an investigation was carried out in order to determine the copper content in blood, organs, samples of seaweeds and sheep foodstuffs. This work is still in progress but some of the preliminary results are as follows:

Seaweeds. The average copper content of oven dried *Rhodymenia palmata* taken in February, March and April was found to be 22 p.p.m., *Alaria esculenta* and *Alaria pylaii* 9.5 p.p.m. Other species of seaweed which the sheep consume contain a varying amount of copper from 3 to 28 p.p.m. It may be concluded that the condition is not caused by too low an intake of copper.

Blood from ewes. The average value for blood copper of ewes from an affected area is less than 0.2 mg Cu per litre. The average blood copper content in ewes from a non-affected area is higher than 1.0 mg Cu per litre.

Livers from lambs. The average copper content of oven dried livers from affected lambs is below 7 p.p.m. which is 20 to 30 times lower than the average copper content of oven dried livers of lambs from non-affected areas.

In order to test the effect of feeding a supplement of copper on the incidence of the disease in lambs a field experiment was carried out during the years 1949-1952. Altogether a group of 1399 ewes on affected farms were given a supplement of copper sulfate, 0.3 g every 10th day during the last 3-4 months of the gestation period. On the same farms 566 ewes were kept as controls.

The ewes which were given the supplement gave birth to 1484 living lambs and of these 35 or 2.3%, showed clinical symptoms of ataxia. The 566 control ewes gave birth to 612 living lambs and of these 101 or 16.3% showed clinical symptoms of the nervous disorder. Stillborn lambs are not included in the figures because of the practical difficulties in bringing the lambs to the laboratory quickly enough for post mortem examination.

In view of the above results, we think that the nervous disorder found in Icelandic lambs born by ewes fed on seaweed during pregnancy is similar to "swayback" or enzoötic ataxia in lambs as described in other countries(1,2).

Summary. 1. A brief description is given of a nervous disorder in lambs born by ewes which feed on seaweeds during pregnancy. Pathologically the disease is characterized by a demyelination confined to the cerebrum. The copper intake in affected areas does not seem to be low, but the ewes in these areas show low values for blood copper, *i.e.* less than 0.2 mg Cu/litre. The copper content of livers from affected lambs is very low. 2. A field experiment showed that the disorder can be largely prevented by giving a supplement of copper to the pregnant ewes.

---

1. Innes, J. R. M., and Shearer, G. D., *J. Comp. Path.*, 1940, v53, 1.

2. Bennetts, H. W., and Chapam, F. E., *Aust. Vet. J.*, 1937, v13, 138.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Growth Characteristics of the Krebs Ascites Tumor. (20403)

HARVEY M. PATT, MARGARET E. BLACKFORD, AND JEAN L. DRALLMEIER.

From the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.

Ascites tumors, which consist essentially of a suspension of neoplastic cells growing in the peritoneal cavity, have been the subject of numerous investigations since the classical observations of Lowenthal and Jahn(1). The suitability of ascites tumors for study of the chemistry and physiology of growth has been emphasized by Klein(2-4), Lettré(5) and others. A number of tumors have been shown to have the capacity for growth as ascites tumors and certain of their chemical and biological characteristics have been described (3,4,7,8). Takes may occur after intraperitoneal injection of a single neoplastic cell (6,9). It is known from earlier work with the Ehrlich ascites tumor of mice that the survival time and body weight increase bear some relationship to the extent of tumor growth. Under certain controlled conditions, survival time is dependent on the number of tumor cells inoculated and the weight increase reflects ascites development(2,4,5,10). While such indices are useful in a general way, a more direct and quantitative measure of the growth characteristics is desirable. This has been attempted in the present experiments by estimating the volume of ascites by dye dilution and the concentration of tumor cells at intervals after intraperitoneal inoculation of the Krebs-2 carcinoma in mice. These studies are preliminary to an investigation of the radiosensitivity of the ascites tumor and related phenomena.

Methods. The ascites tumor* employed in these experiments was derived originally from the Krebs-2 solid carcinoma. The Krebs ascites tumor is closely related cytologically and chemically to the Ehrlich ascites tumor (11). Female CF-1 mice 10 to 12 weeks of age were injected intraperitoneally with 0.2 ml of freshly obtained ascitic fluid diluted with Tyrodes solution to give the desired tumor cell concentration. Animals were sacrificed by cervical fracture at intervals after tumor inoculation. Evans blue dye, usually 1.5 ml of a 0.05% solution, was injected i.p. immediately after sacrifice, care being taken to inject the entire volume directly into the peritoneal cavity. The dye solution was prepared in cell free ascitic fluid obtained by high speed centrifugation and repeated freezing and thawing. Two minutes were allowed for mixing of the dye and ascitic fluid by gently rotating the animal before an aliquot was removed with a glass pipette through a small incision in the peritoneal wall. This interval was adequate for complete mixing. The sample was divided into 4 portions: (1) for determination of the percentage of cell free fluid by centrifugation in Wintrobe tubes, (2) for measurement of dye dilution on the cell free supernate, (3) for estimation of cell concentration in a hemocytometer, (4) for preparation of air-dried smears stained with Feulgen reagent and methyl green. The degree of dye dilution was determined spectrophotometrically at 620 μ on a 1:25 dilution of the cell free supernate. The dye concentration was determined in the usual manner by comparison with the transmission of standard solutions; the latter was remarkably constant from one experimental period to another. Since dye uptake by the cells during the several minutes between injection and centrifugation is negligible, dye dilution is a direct measure of the volume of ascitic plasma. The total volume of ascites may be calculated from the dye dilution and the percentage of cell free fluid.

---

* Kindly provided by Dr. T. S. Hauschka.

$$\text{Ascites in ml} = \frac{100\,(C_i/C_d)\ (\text{ml dye})}{\%\ \text{cell free fluid}} - \text{ml dye inj.}$$

where C_i = dye concentration inj.
C_d = dye concentration determined.

The error of this procedure, estimated from injection of known volumes of ascitic fluid (2-5 ml) and their recovery by dye dilution, is 4.9 ± 1%. Cell counts were made in a Spencer Bright Line hemocytometer using a binocular microscope and a magnification of 270. For this purpose, 0.1 ml of cell suspension was diluted with a suitable volume of Tyrodes solution containing 1% Eosin Y (National Aniline). Resistance to eosin-staining may be assumed to be an index of viability(3,12,13). Determinations were made in duplicate and at least 1000 cells, exclusive of erythrocytes, were counted in each instance. It was possible from the wet counts to classify the cells both as to size (greater or less than ca 12 μ in diameter) and eosin resistance. An attempt was made to differentiate the various cell types on the Feulgen-methyl green stained smears. Differential counts were made in duplicate; a minimum of 1000 cells was enumerated on each smear using a magnification of 540. Cells were classified as tumor cells, polymorphonuclear leucocytes, lymphocytes, large nontumor cells (presumably mainly mesothelial cells and histiocytes), and small cells other than polymorphonuclear leucocytes and lymphocytes. The frequency of tumor cells in mitosis was also determined. Although there are no absolute cytological criteria to distinguish a tumor cell from a nontumor cell, it was possible to differentiate the neoplastic cells with reasonable reproducibility from one slide to another on the basis of cell size and the large and variable nucleus with one or more large nucleoli. These cells are present in abundance relative to the other cell types and differ from the cells found in normal or inflammatory peritoneal fluid.

Results. The tumor cell population increases exponentially with a doubling time of 19 to 20 hours during the first 3 days after inoculation of 20 x 10^6 cells. After the initial log phase, the total number of tumor cells suspended in ascitic fluid increases more slowly and approaches an asymptote of about 10^9 cells at one week. These data are depicted in Fig. 1, which also reveals the close correspon-

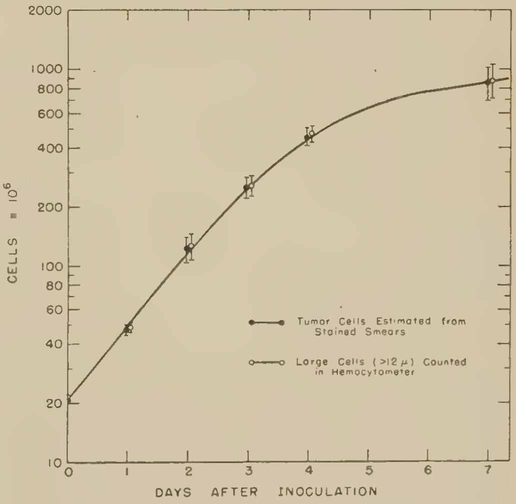


FIG. 1. Number of tumor cells as a function of time after inoculation. (Each value represents the mean of duplicate determinations on 5 mice; vertical lines designate stand. error of the mean.)

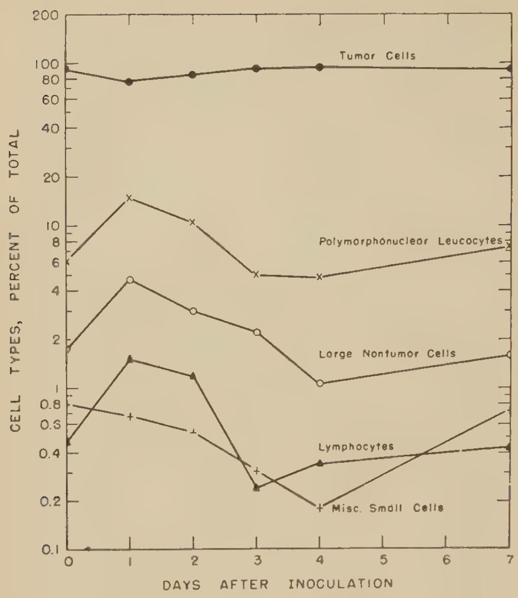


FIG. 2. Changes in composition of ascites after inoculation of 20 × 10^6 Krebs-2 tumor cells. (Each value represents the mean of duplicate determinations on 5 mice.)

dence between tumor cells estimated from the Feulgen-stained smears and the large cells (> ca 12 μ) counted in the hemocytometer. The percentage of large eosin-stained cells is relatively constant during the entire course of tumor development. The frequency of mitosis ranges from 2 to 2.8% during the period of logarithmic growth and is 1.2% seven days after tumor cell inoculation. The period of slowed growth or leveling corresponds to the time of death of mice inoculated with 20 x 10^6 Krebs ascites tumor cells (50% fatalities at 5 days; 100% fatalities at 10 days).

Differentiation of the various cell types is shown in Fig. 2 where it will be noted that the percentage of tumor cells in the total population is decreased from the inoculum value of 92% to 78% on the first day. The decrease in relative tumor cell concentration corresponds to the apogee of an inflammatory reaction as manifested by an increase in polymorphonuclear leucocytes, lymphocytes, and large nontumor cells, presumably mainly mesothelial cells and histiocytes. The percentage of tumor cells returns to a rather constant level of about 90% on the third day, which coincides with the decline of the inflammatory cells. The differential cell counts at 7 days are remarkably similar to the cell population of the original inoculum.

The volume of ascitic fluid increases progressively during the course of tumor development. In general, the rate of increase parallels that of the tumor cells during the initial log period, but exceeds the latter during the more terminal period (Fig. 3). Body weight is also increased during tumor growth, but this parameter is apparently a more complex function of the ascites and its sequelae. The data are summarized in Table I.

Discussion. The methods employed in these studies provide a convenient and reasonably quantitative measure of certain of the growth characteristics of the ascites tumor. The problems inherent in the diagnosis of individual tumor cells in general and in ascitic fluids in particular have been discussed by Klein(3). Since the Krebs-2 ascites consists of an almost pure culture of cells which differ from the cells normally found in peritoneal exudates, the over-all errors in discrimination may be comparatively small. Diagnoses of duplicate smears in the present experiments agree within 5%. The close correspondence between tumor cells estimated from the stained preparations and large cells enumerated in the hemocytometer is of interest in this connection. Since each determination was independent of the other, the wet counts may suffice for many routine purposes. While differential counts provide useful information relative to tumor development, the additional parameter of ascitic volume enables a more

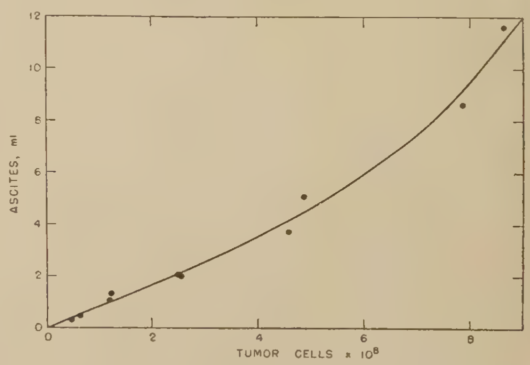


FIG. 3. Relationship between number of tumor cells and volume of ascites. (Each value represents the mean of 5 mice.)

TABLE I. Number of Cells, Ascitic Volume and Body Weight as a Function of Time after i.p. Inoculation of 20 × 10^6 Krebs-2 Tumor Cells. (Each value represents the mean ± S.E. of 11 mice except for % mitosis, based on 5 mice each.)

Days after inoculation	Total cells × 10^6	Tumor cells × 10^6	Tumor cells % eosin stained*	Tumor cells % mitosis	Ascitic vol, ml	Body wt increase, g
1	73.4 ± 4.7	57.2 ± 3.9	1.6 ± .24	2.2 ± .15	.4 ± .04	−.1 ± .21
2	142.3 ± 10.9	120.9 ± 9.9	2.0 ± .31	2.8 ± .17	1.2 ± .16	1.4 ± .40
3	266.7 ± 23.4	250.4 ± 22.2	2.0 ± .27	2.0 ± .23	2.0 ± .20	5.0 ± .19
4	507.0 ± 34.1	474.6 ± 31.6	1.7 ± .18	2.0 ± .16	4.5 ± .40	9.2 ± .20
7	1010.2 ± 98.0	913.6 ± 87.5	2.8 ± .61	1.2 ± .18	10.2 ±1.35	11.7 ±1.5

* Based on large cell counts in hemocytometer.

complete analysis of the growth function. The conventional dye dilution technic is applicable in this instance. The injection of dye solution prior to sampling has the additional advantage of providing a more uniform aliquot for analysis, particularly during the first day or two after tumor inoculation.

The data reveal that ascites tumor development after inoculation of 20 x 10^6 cells consists of an initial log phase of several days duration followed by a period of less rapid growth during which the cell population approaches an asymptote. Estimates of the mitotic time (25 to 30 minutes) based on the mitotic index and the interval required for a doubling of the cell population during the log phase agree with the values reported for other types of cells(6). The apparent decrease in rate of growth with time may be related to the moribund state of the animals. It may also be recalled that the mitotic index is decreased during this period. It is likely, moreover, that the number of cells removed from the effective ascitic pool by adherence or organized growth on peritoneal surfaces increases with time and the magnitude of the ascites(4,10). Qualitatively-similar growth curves have been observed with a less concentrated inoculum, *e.g.*, 1 x 10^6 tumor cells. In this instance the log phase persists for about one week and the period of decelerated growth commences when the development of ascites is comparable to that observed with the more concentrated inoculum. It is not known whether a brief lag or latent period intervenes during the first hours after tumor inoculation. This may perhaps be resolved by inoculating a large volume of undiluted ascites initially to minimize sampling errors due to the small amount of fluid formed during the first day, or by re-inoculation of the same animals during the midpoint of logarithmic growth.

The differential cell counts are suggestive of a transient inflammatory reaction during the first day or two after inoculation. In general, a similar host reaction has been described after intraperitoneal injection of several varieties of ascites tumors and of cell-free ascitic fluid(3,4,7). An important difference in the relative frequency of the tumor cells may be noted, however. A pronounced decrease in the percentage of Ehrlich ascites tumor cells (from an initial value of about 80% to less than 5%) has been observed during the first 24 hours after inoculation(3). This is followed by an almost equally abrupt increase in tumor cell frequency on the second day. The present experiments with the Krebs ascites differ markedly in the extent of the initial decline in the relative frequency of tumor cells even though the values from 3 days on are in essential agreement. The initial discrepancy may be due in part to the difficulty in differentiating tumor cells from the large mononuclear cells during the inflammatory reaction and possibly to the host response, which is known to differ from one strain to another(3,4). It is unlikely, however, that these factors are mainly responsible for differences of this order. It may be recalled that the volume of ascites is quite small during the first 24 hours and it is well, therefore, to consider the methods of sampling in the two cases. It seems reasonable to assume that the prior injection of Evans blue solution and subsequent mixing by rotation of the animal will assure a more uniform sample for withdrawal than simple puncture and aspiration of the ascites during the early growth period. The injection procedure, employed in the present experiments, should serve also to free tumor cells adhering to peritoneal surfaces because of the small volume of ascites. Klein(3) has, in fact, intimated that a mechanism of this sort may account for part of the large decrease in relative numbers of tumor cells observed by him during the first 24 hours. These considerations are obviously germane to the question of the early fate of the injected cells and, therefore, to the matter of a lag period in tumor development.

Ascitic fluid volume appears to be a linear function of the number of tumor cells during the log phase, but increases somewhat more rapidly during the terminal period. Ascitic fluid volumes of 20 ml or more are not uncommon during the second week of tumor growth. Ascitic fluid obtained from mice with the Ehrlich tumor contains 2.5 to 3% protein, about 60% of which is albumin(14). The absorption spectrum of the Krebs fluid resembles that of the Ehrlich ascites. Fluid obtained during the first few days is usually free of erythrocytes; however, a bloody ascites is frequent by the end of the first week and may be correlated with peripheral anemia. The results are generally suggestive of an effect on capillary permeability by the products of tumor cell metabolism or breakdown, but this remains to be demonstrated. Edema of skin and muscle is evident several days after tumor inoculation and may perhaps be attributed to the loss of plasma protein and consequent decrease in oncotic pressure. There are also indications of an absolute decrease in dry weight of the whole animal during the later stages of ascites development. The nature of the relationships between body weight, the volume of ascites and the number of tumor cells will be reported elsewhere.

Summary. Experiments are reported in which the growth of an ascites tumor in mice has been followed by estimating the volume of ascites by dye dilution and the concentration of tumor cells from total and differential cell counts. The methods afford a reasonably quantitative picture of the growth pattern, which, after inoculation of 20 x 10^6 Krebs-2 ascites tumor cells, consists of an initial log phase of several days duration followed by a period of leveling to an asymptote of about 10^9 cells. The period of apparent less rapid growth parallels the moribund condition of the animals. The number of tumor cells doubles in 19 to 20 hours during the log phase. The mitotic index is around 2.3%, which is suggestive of a mitotic time of 25 to 30 minutes. It is not possible from these experiments to determine the extent, if any, of a lag or delay period in growth immediately after tumor inoculation. The volume of ascites and body weight increase progressively during the course of tumor development. However, the relationships between these parameters and the tumor cell population apparently depend on a number of factors. The results are discussed in connection with related observations on the growth characteristics of ascites tumors.

---

1. Lowenthal, H., and Jahn, G., *Z. Krebsforsch,* 1932, v37, 439.
2. Klein, G., *Cancer,* 1950, v3, 1052.
3. ———, *Exp. Cell Research,* 1951, v2, 518.
4. ———, *The Production of Ascites Tumors in Mice and Their Use in Studies on Some Biological and Chemical Characteristics of Neoplastic Cells,* 1951, Uppsala Almquist and Wiksells, Boktryckeri AB, 32p.
5. Lettré, H., *Z. Physiol. Chem.,* 1941, v268, 59.
6. Yoshida, T., *J. Nat. Cancer Inst.,* 1951-52, v12, 947.
7. Goldie, H., and Felix, M. D., *Cancer Res.,* 1951, v11, 73.
8. Levan, A., and Hauschka, T. S., *Hereditas,* 1952, v38, 18.
9. Hauschka, T., *Proc. Am. Assn. for Cancer Res.,* 1953, v1, 24.
10. Lettré, H., *Z. Krebsforsch,* 1950, v57, 1.
11. Klein, G., and Klein, E., *Cancer Res.,* 1951, v11, 466.
12. Schrek, R., *Am. J. Cancer,* 1936, v28, 389.
13. Straube, R. L., Svihla, G., Blackford, M. E., and Patt, H. M., *Am. J. Physiol.,* 1951, v167, 829.
14. Kun, E., Talalay, P., and Williams-Ashman, H. G., *Cancer Res.,* 1951, v11, 855.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Protoporphyrin Production of *Tetrahymena geleii.* (20404)

M. A. Rudzinska* and S. Granick. (Introduced by P. K. Olitsky)
From the Laboratories of The Rockefeller Institute for Medical Research, New York City.

1. *Growth of the organism and formation of the pigment.* Cultures of *Tetrahymena geleii,* strain W,† which had been grown for many years in daylight as a food for *Tokophrya infusionum*(1,2) were observed about a year ago to produce a red pigment when grown in the dark. The cultures were grown in test tubes at 25°C on an agar medium containing 2% proteose peptone, 1.5% agar, 0.25% dextrose, 0.025% $(NH_4)_2HPO_4$ and 0.0005% thiamin. The pigment in Tetrahymena was observed, by microscopical examination, to be enclosed in small vacuoles about 3-4 μ in diameter. The greatest accumulation of vacuoles with red pigment was located in the posterior part of the body. It was observed also that some of the pigment, still enclosed in a vacuole, was excreted from the body through the cytopyge. Once outside the body of the Tetrahymena the pigment collected at the bottom of the water of condensation in the test tubes and also along the surface of the slant where the agar, water of condensation, and the inner side of the tube meet. Individuals containing the red pigment were found to be of greater size than those without it.

Growth on a solid medium appears to be necessary for the production of the pigment. In liquid medium the red pigment was absent from the organism even when a culture with organisms containing the red pigment was used as inoculum for subcultures in a liquid medium. However, the occurrence of individuals with red pigment was greater in subcultures made from cultures grown in a liquid medium than in those transferred from a solid medium to another solid medium. The number of organisms containing red pigment was greatest in a culture 5-8 days old. As the cultures became older the color turned somewhat brownish. The red pigment when excited by U.V. light of 400 mμ fluoresced red, a property of porphyrin compounds. The younger and older cultures appeared to contain only one major pigment, identified subsequently as protoporphyrin.

Fluorescing porphyrin pigments are known to be photodynamically active(3) and in the presence of visible light might be expected to be inhibitory to growth. Very dilute protoporphyrin solutions have been reported by Fischer and Schneller(4) to act as photosensitizers in *Paramecium.* For example, when a few drops of this diluted porphyrin were added to cultures of *Paramecium* and the cultures then exposed to sunlight all the organisms were killed within 10 minutes, whereas control cultures with the same amount of porphyrin, when left in the dark, remained unaffected.

In our experiments we have grown cultures of Tetrahymena in the presence and absence of light. When cultures were grown on agar for 4 days in continuous light, growth was diminished and no pigment appeared. When given 12 hours light and 12 hours dark, growth was better and no red pigment appeared. However, when the organisms were grown in the dark growth was best and the pigment was abundant. Cultures containing organisms with red pigment were exposed to continuous or intermittent light. After a few days no red organisms could be found but only red pigment in the medium. In the control cultures kept in the dark, organisms containing the red pigment were still abundant. It seems that absence of light favors growth of organisms with red pigment.

2. *Isolation and identification of the pigment from Tetrahymena.* Reddish and red-brown materials which had been obtained from Tetrahymena grown in test tubes, as described above, were examined and as far as could be determined the color of these small amounts of pigment appeared to be due to

---

* Supported by a grant from the National Heart Institute.

† Acknowledgement is made to Dr. R. P. Hall for his courtesy in supplying the *Tetrahymena geleii* W.

protoporphyrin. To obtain more of the pigment, Tetrahymena was grown in 3 Blake flasks. Each flask contained one liter of the agar medium described above supplemented with 0.1% Bacto yeast extract. After 10 days growth in the dark, the agar surface appeared to be somewhat brownish in color. The surface of the agar was washed with water and the suspension which was obtained was centrifuged. Two layers separated out at the bottom of the centrifuge tube, a thick white lower layer containing the cells, and a thinner red-brown upper layer containing pale pinkish spherical bodies 3-4 μ in diameter evidently derived from the cells. The supernate which contained no porphyrin was discarded.

The upper red-brown layer was separated from the lower layer by gently suspending it in a small amount of acetone and decanting it. The red-brown suspension was extracted first with acetone and then several times with 10 cc volumes of acetone-HCl 100:1 (v/v). The acetone-HCl solution was transferred to ether and fractionated by extracting successively with increasing concentrations of aqueous HCl. Two porphyrin fractions were obtained. Most of the porphyrin was extractable with 0.5-1.0 N HCl which suggested that this was protoporphyrin. About one tenth of the total porphyrin could not be extracted from ether solution with 1 N HCl although it appeared to have the absorption spectrum of protoporphyrin. This latter porphyrin became extractable only when the acidity was increased to 8 N HCl. However, once the porphyrin became soluble in the aqueous phase it came to have the HCl number and absorption spectrum of protoporphyrin. From these properties it may be inferred that a fraction of the total protoporphyrin in Tetrahymena is bound to some fatty material perhaps in ester linkage. Treatment with 8 N HCl appears to split this linkage.

A study of the absorption spectra of the 2 porphyrin fractions supported the identification of this porphyrin as protoporphyrin. With a Cary spectrophotometer determinations were made on the porphyrin, of the positions of the absorption maxima in ether solution and the ratios of the absorption maxima to each other. The positions of the maxima and the ratios agreed with those found for crystalline protoporphyrin. From the density of absorption it is estimated that a total of 0.6 mg of protoporphyrin was obtained from the Tetrahymena culture of the 3 Blake flasks. By a paper chromatography method further support was obtained for the identification of the porphyrin as protoporphyrin.

3. *Discussion.* Free protoporphyrin in contrast to its iron complex, heme, has been reported to occur normally in relatively large amounts only in the Harderian gland of the genus *Mus.* In all other instances, protoporphyrin accumulates due to some unknown metabolic lesion, as in the case of a mutant *Chlorella*(5,6) or in porphyria cutanea tarda of humans(7). It is interesting to note that in the case of the *Chlorella* mutant as well as in the present *Tetrahymena geleii* W strain, protoporphyrin is formed most readily on solid media in the dark and generally in older cultures. Heme is present in Tetrahymena as determined by the pyridine hemochromogen test so that at least qualitatively iron can be inserted into the protoporphyrin ring by the organism. The finding of the red pigment outside the cells suggests that the cells containing the pigment may excrete it or may break open more readily than cells not containing the pigment.

4. *Summary.* A strain of *Tetrahymena geleii* W at the end of its rapid phase of growth on solid media in darkness turns red-brown. The main pigment component has been identified as protoporphyrin. A minor component containing protoporphyrin appears to be bound to some fatty material.

---

1. Rudzinska, M. A., *Science,* 1951, v113, 10.
2. ———, *Biol. Bull.,* 1951, v100, 49.
3. Blum, H. F., *Physiol. Rev.,* 1932, v12, 23.
4. Fischer, H., and Schneller, K., *Z. physiol. Chem.,* 1923, v130, 302.
5. Granick, S , *J. Biol. Chem.,* 1948, v172, 717.
6. ———, *Ann. Rev. Plant Physiol.,* 1951, v2, 115.
7. Remington, C., *Acta Medica Scand.,* 1952, v143, 161.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Isolation of Penicillin-Susceptible Mutants from Penicillinase-Producing Strains of *M. pyogenes*.* (20405)

AMEDEO BONDI, JOAN KORNBLUM, AND M. de SAINT PHALLE.
From the Department of Bacteriology, Hahnemann Medical College, Philadelphia, Pa.

Recent reports(3,5) indicate that the incidence of penicillin-resistant strains of *Micrococcus pyogenes* causing infections of man is increasing. Many of the factors responsible for the emergence of such a resistant population of micrococci are as yet unknown. It is a well established fact(1,2,4), however, that the ability to produce the enzyme, penicillinase, is the primary factor in the natural resistance of these cultures of *M. pyogenes* to penicillin. Of considerable interest in this respect is the report of Barber(6) indicating that penicillin-sensitive mutants may be isolated from cultures of penicillinase-producing micrococci. The sensitive mutants described by this worker did not produce the enzyme. The study reported in this paper was undertaken to investigate this phenomenon further in an attempt to determine its possible relationship to the problem of the increasing incidence of penicillin-resistant micrococci.

Materials and methods. The strains of micrococci studied consisted of a group of penicillinase-producing cultures isolated from infections over a period of the past 5 years. Since there is no correlation between penicillinase-production and type of pigment(7), we have not classified these strains as *Micrococcus pyogenes* var. *aureus* or *Micrococcus pyogenes* var. *albus*. All cultures were maintained on brain heart infusion (B.H.I.) agar slants covered with sterile mineral oil. During the period of investigation, stock cultures of each strain were transferred monthly on B.H.I. agar slants and stored in the refrigerator. Unless otherwise noted, nutrient agar and broth were used exclusively throughout the study. During the early phases of this study, the method for detecting susceptible mutants was similar to that of Barber(6) with minor modifications. Cultures of penicillinase-producing micrococci to be tested were inoculated into large tubes of broth, 30 ml per tube, and incubated at 37°C for 24 hours, and subsequently stored at room temperature. These cultures were plated out on the surface of agar plates at various intervals, ranging from one day to several months. After suitable incubation, 50 or more colonies were picked and single streaks of each made across the surface of an agar plate. As many as 13 colonies could be tested conveniently on a single plate. Strips of sterile filter paper approximately ¼″ x 3″ saturated with penicillin, 50 units/ml, were placed across the plate perpendicular to the lines of inocula. Staphylococcus H, a known susceptible strain of *M. pyogenes* var. *aureus*, was likewise included as a control. The inocula from resistant wild-type colonies grew luxuriantly adjacent to the penicillin-saturated strip of filter paper; the inocula from sensitive mutant colonies showed zones of inhibition similar to that of the control strain. Although this method, designated as the "stripping procedure", is highly accurate as a method for detecting susceptible colonies, only a small number of cells could be tested at one time. During the course of this study, a more selective method was developed, therefore, for the detection of non-penicillinase-producing mutants. In this procedure the penicillinase-producing culture to be tested was plated on tetrazolium agar plates of the following composition: .5% tryptose, .5% proteose peptone, .4% beef extract, 1.0% dextrose, .5%NaCl and 2% agar. The medium was adjusted to pH 7.5 and sterilized in the autoclave at 15 pounds pressure for 15 minutes. Triphenyltetrazolium, .0025%, and penicillin, .025 unit/ml were added aseptically to the melted and cooled agar. On such plates, the parent wild type colonies in 48 hours are pink, glistening and convex; colonies of the sensitive mutants are paler pink, flattened, and have one or two raised concentric rings and small central dark spots. The two types of colonies

---

* This investigation was supported by a research grant, E154, from the National Institutes of Health, U. S. Public Health Service.

TABLE I. Incidence of Penicillinase-Negative Mutants Isolated from Penicillinase-Producing Strains of *M. Pyogenes.*

	Stripping procedure			Tetrazolium method		
	Colonies	S mutants		Colonies	S mutants	
Strain	tested	No.	%	tested	No.	%
7170	133	6	4.5			
163	291	1	.3			
7739	154	4	2.6			
" SM resist.	1109	6	.5	39127	126	.3
7701	100	2	2.0			
7729	256	192	75.0	1797	1410	78.0
2255	150	1	.7			
161	482	0	0	13625	0	0
446	181	20	11.0	3147	317	10.1
D-3	185	3	1.6	1183	23	1.9
1215	261	17	6.5			
1175*	169	14	8.3			
7970	224	39	17.4	3489	631	18.1
7791	243	1	.4	2519	21	.8

* This strain was completely inhibited on the tetrazolium agar plates.

are readily differentiated making possible the evaluation of 200 to 400 colonies on a single plate. The mechanism of the reaction which makes possible the separation of the two types of colonies is presently under investigation. The susceptibility to penicillin of a suspected mutant colony of *M. pyogenes* was confirmed by the disk test, and when indicated by the tube dilution method. This was sufficient evidence of this organism's inability to produce penicillinase in view of the direct correlation between its susceptibility to penicillin and its inability to produce the enzyme (1,2,4). In this paper, colonies from penicillinase-producing cultures which do not produce the enzyme are designated S mutants.

Experimental results. Thirteen strains of penicillinase-producing micrococci were studied to determine their ability to give rise to non-penicillinase-producing mutants. Cultures of the strains were tested after different periods of incubation. On each trial a minimum of 50 colonies was selected at random and tested by the "stripping procedure." For comparison certain of the cultures were likewise tested by the tetrazolium agar plate method in which case only 48 hr broth cultures were tested. As shown in Table I, 12 of the 13 cultures tested produced S mutants. In general the two methods compare rather favorably as indicated by the mutation rates for those cultures tested simultaneously by both methods. The tetrazolium agar plate method, however, has the advantage of permitting the testing of much larger populations of cells. The results indicate that the mutation in question occurs at a relatively high rate of frequency although the rate varies considerably from one strain to another.

To eliminate the possibility that S mutants were contaminants, a streptomycin resistant (SMR) mutant of *M. pyogenes,* strain 7739, was included as an additional control. As indicated in Table I, penicillinase-negative colonies were also isolated from this SMR strain. These mutants were likewise streptomycin resistant. The improbability of isolating spontaneously such a double mutant (non-penicillinase-producing and streptomycin-resistant) makes less likely the possibility that the mutants isolated were contaminants. In all further work, whenever possible, the SMR strain of *M. pyogenes* 7739 was used. Further evidence that the mutants isolated were true mutants was obtained by phage typing.[†] Similar patterns of lysis were given by wild type and mutant cultures. Repeated passage of S mutants always yielded 100% sensitive colonies. Evidence of reverse mutation has not been detected following repeated subculture over a period of two years. The susceptibility of the mutant strains to penicillin is quite comparable to that of the known sensitive cultures of *M. pyogenes,* namely strains 209 P and H as shown in Table II.

[†] We are indebted to Dr. John Blair, New York City for the typing of these strains.

TABLE II. Penicillin Susceptibility of Penicillinase-Negative Mutants of *M. pyogenes* 7739 SMR.

Strain	Penicil-linase	Inoculum, 0.1 ml	M.I.C.,* units/ml
7739	+	10^{-1}	25.0
		10^{-4}	.4
7739 S_1	—	10^{-1}	.1
		10^{-4}	.05
7739 S_2	—	10^{-1}	.1
		10^{-4}	.03
7739 S_3	—	10^{-1}	.1
		10^{-4}	.05
H	—	10^{-1}	.05
		10^{-4}	.013
209 P	—	10^{-1}	.1
		10^{-4}	.05

* M.I.C. = Minimal inhibiting concentration.

TABLE III. Incidence of S Mutants Isolated from *M. pyogenes* 7739 SMR Grown under Various Cultural Conditions.

Cultural condition	No. colonies evaluated	S mutants	
		No.	%
Aerobic control	5345	55	1
Anaerobic*	2498	19	.8
5% dextrose	1364	4	.3
.6% HCOH†	974	11	1.1
.04% $MnCl_2$†	1842	20	1.1
5% Na desoxycholate†	1597	36	2.2
.001% hydroxyquinoline	2504	8	.3
.08% NaN_3	3752	99	2.6
Erythromycin‡	2173	4	.2
Magnamycin‡	3092	8	.26
Terramycin‡	1724	5	.3
Chloromycetin‡	4617	200	4.3
Aureomycin‡	1906	14	.7
Growth at °C			
23	2965	36	1.2
30	1445	23	1.6
37	910	17	1.9
45	4385	1081	24.6
pH 5.85	3323	43	1.3
7.56	3365	22	.6
8.10	2836	30	1.1
Ultraviolet irradiation:			
Zero point	1426	26	1.8
End "	1227	35	2.8

* Method of Spaulding and Goode(10).
† Tested against resting cells.
‡ The maximum sub-inhibiting concentration of each.

Studies were carried out to determine factors which might influence the rate of mutation. Cultures of *M. pyogenes* 7739 SMR were grown under a variety of conditions for 48 hours and tested for S mutants by the tetrazolium agar method. Many of the cultural conditions were selected because they were known to induce various types of mutations. Others were chosen on the basis of preliminary evidence that the mutation rate might be higher under certain conditions(11) or that certain agents(8,9) might influence penicillinase production. The results of these experiments are tabulated in Table III. Most of the cultural conditions studied did not significantly influence the mutation rate. The outstanding exception, however, is cultivation of the strain tested at 45°C. At this temperature, the mutation rate was increased 20 fold. Preliminary studies indicate that this increased rate is not due to a selective action of the higher temperature.

Discussion. The sensitive mutants of penicillin-resistant strains of *M. pyogenes* as described in this report appear to be identical with those described by Barber(6). We were successful in isolating sensitive mutants from 12 of 13 (92%) strains tested whereas Barber found only 17 of 32 (53%) cultures to be positive. Our success in this respect may be attributed to the testing of larger populations of cells. Barber(6) likewise reported a high rate of mutation which varied considerably from one strain to another. Our failure to detect S mutants in strain 161 does not necessarily indicate that they are not produced by this strain. The mutation rate could be extremely low or the S mutants of this strain might be inhibited on the tetrazolium agar plates. Such inhibition was observed (Table I) with strain 1175 which produced S mutants when tested by the "stripping procedure".

The high incidence of S mutants in strain 7729 is not surprising. Barber(6) showed that certain of her strains had similar high rates of mutation. Of particular interest in regard to strain 7729, however, is the fact that when tested two years previously it had a much lower incidence of S mutants. This observation would indicate that in the case of this strain certain factors as yet unknown influence the mutation rate.

Further study is necessary before one can evaluate the significance of these susceptible mutants of *M. pyogenes*, or determine their relationship to the increasing incidence of resistant strains. It is interesting to speculate as to the possibility of this mutation occurring *in vivo*. Preliminary attempts to isolate non-penicillinase-producing mutants from other penicillinase-producing organisms such as *E. coli* and *Shig. paradysenteriae* were unsuccessful. If non-enzyme-producing mutants are produced by these gram-negative bacilli, the frequency with which they occur is considerably lower than that of *M. pyogenes*.

Summary. Twelve of 13 strains of *M. pyogenes* which produce penicillinase yielded non-penicillinase-producing mutants which were susceptible to penicillin. This apparent spontaneous type of mutation occurs at a high rate of frequency. Cultivation of a strain of this organism at 45°C significantly increased the number of mutants. No evidence of reversibility of the mutation was obtained.

---

1. Bondi, A., and Dietz, C. C., *J. Bact.*, 1948, v55, 843.

2. ———, Proc. Soc. Exp. Biol. and Med., 1944, v56, 135.

3. Barber, M., and Rozwadowska-Dowzenko, M., *The Lancet*, 1948, v2, 641.

4. Spink, W. W., *J. Lab. and Clin. Med.*, 1951, v37, 278.

5. Beigelman, P. M., and Rantz, L. A., *New Eng. J. Med.*, 1950, v242, 353.

6. Barber, M., *J. Gen. Microbiol.*, 1949, v3, 274.

7. Bondi, A., and Dietz, C. C., Proc. Soc. Exp. Biol. and Med., 1945, v60, 55.

8. Woodruff, H. B., and Foster, J. W., *J. Bact.*, 1945, v49, 7.

9. Dietz, C. C., and Bondi, A., *J. Bact.*, 1948, v55, 849.

10. Spaulding, E. H., and Goode, W. G., *J. Lab. Clin. Med.*, 1939, v25, 305.

11. Chandler, C. A., Davidson, V. Z., Long, P. H., and Monnier, J. J., *Bull. J. Hopkins Hosp.*, 1951, v89, 81.

Received June 9, 1953. P.S.E.B.M., 1953, v83.

Effect of Diet on Rate of Alcohol Oxidation by the Liver.* (20406)

Elaine Kerner, and W. W. Westerfeld.

From the Department of Biochemistry, State University of New York, Medical College at Syracuse.

Many studies on the effects of alcohol have been conducted with little attention being given to other details of the diet. This is particularly true when relatively large amounts of alcohol are administered to animals who are otherwise consuming an adequate diet, for under these circumstances the total caloric intake is not increased, the amount of food eaten is decreased, and the consumption of essential nutrients may be reduced to a deficiency level. Effects which are attributed to alcohol may in fact be due to a concomitant nutritional deficiency (such as protein) produced by the substitution of alcohol for food. The present studies were conducted to determine how much the oxidation of alcohol itself by the liver might be influenced by protein depletion or inanition.

Experimental. The oxidation of alcohol was measured manometrically.† Livers were removed from decapitated rats, and weighed portions were homogenized with 4 volumes of 0.04M phosphate buffer, pH 7.4. Two pairs of Warburg flasks each contained 1 ml of the homogenate and 0.2 ml of 0.1M semicarbazide hydrochloride; one pair of flasks contained 0.2 ml of 15% ethyl alcohol (by volume) in the side arm. Water was added to all flasks to give a total volume of 2.0 ml, and an additional 0.2 ml of 10%KOH plus a filter paper were placed in the center wells. The flasks were equilibrated at 37° for 10 minutes, the zero reading was taken, and the alcohol was tipped in from the side arm. Readings were taken thereafter at 10-minute intervals for 80 minutes. The increased oxygen consumption in the alcohol-containing flasks was obtained by subtracting the endogenous respiration in the control flasks, and the net oxygen

---

* This study was aided by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

† This method is similar to the one used by Dr. Victor A. Najjar, to whom we are indebted for supplying the details prior to their publication.

TABLE I. Effect of Diet on Ability of Rat Liver to Oxidize Alcohol.

Rats	Diet	Days on diet	Body wt		No. of rats	Alcohol oxidation by liver homogenate*
			Start	End		
Weanlings	As received	—	—	44	5	12 ± 2.0
	Chow	16	46	109	6	21 ± 1.2
	Purified 24% casein	16	49	80	5	18 ± 2.0
	Purified protein-free	16	43	31	7	3 ± 0.6
Adults	Chow	24	375	400	9	24 ± 1.2
	Purified 24% casein	24	369	407	10	19 ± 1.0
	" protein-free	24	378	302	10	4 ± 0.4
"	Starvation	7	216	156	12	11 ± 1.0
	50% restriction of chow	14	234	161	10	20 ± 0.9

* Alcohol oxidation values have been recorded as the net O_2 consumption in mm^3 per 10 min. per Warburg flask containing 200 mg fresh liver; mean ± stand. error of mean.

consumption due to alcohol was plotted against time. The rate of alcohol oxidation was taken from the straight-line portion of this curve, and has been recorded as cmm of oxygen uptake per 10 minutes per flask containing 200 mg fresh liver. The straight-line portion of the curve usually covered the first 2 to 6 readings, though with some livers of intermediate activity there was a slower rate or lag period during the 1st 10 or 20 minutes.

This procedure was designed to be a measure of the alcohol dehydrogenase in liver, but it has not been so labeled because there is no assurance that other components of the liver or other simultaneous reactions might not also affect the rate of oxygen uptake. Semicarbazide was added to the flask in order to bind the acetaldehyde and thereby limit the oxidation of the alcohol to its initial step; semicarbazide had no effect on the endogenous respiration and may have increased slightly the rate of oxygen consumption in the presence of alcohol. Since the transfer of hydrogen from the substrate, alcohol, to oxygen of the air involved numerous intermediate steps, and since any one of these steps could be limiting the rate of oxygen consumption, the effect of other additions was tested. Methylene blue, nicotinamide or DPN were without beneficial effect and may have been inhibitory. A washed heart muscle preparation as a source of the cytochromes was without effect. The simultaneous addition of DPN, cytochrome C and the washed heart muscle homogenate had no effect on the net rate of oxygen consumption with alcohol as the substrate, and this was true for the livers of low alcohol oxidative capacity obtained from protein-depleted rats as well as for normal livers. Hence there was no indication that the procedure measured anything but alcohol dehydrogenase, but there was also no positive evidence obtained on this point.

The effect of various diets on the ability of the liver homogenate to oxidize alcohol was studied in both weanling and adult rats. Some of the male weanling rats were analyzed upon receipt from Albino Farms; others were fed a diet of: 1) chow, 2) purified 24% casein, or 3) a protein-free diet for two weeks before analysis. Adult male hooded rats were divided into 3 groups and fed the same 3 diets for 24 days before analysis. In addition, the effect of caloric restriction was tested in the adult rats by completely starving one group for 7 days, and by allowing each rat in another group to eat only 10 g of chow daily; this was 50% of the *ad libitum* intake measured during the previous week, and was continued for 14 days before the rats were sacrificed.

The purified 24% casein diet contained (in g): Labco Casein 24, Crisco 4, Wesson oil 2, cod liver oil 1, Phillips and Hart salts 4, glucose 65, plus a vitamin supplement (in mg per 100 g of diet) of: choline 100, niacin 2.5, calcium pantothenate 1.0, riboflavin 0.4, thiamine 0.4 and pyridoxine 0.4. The protein-free diet had the same composition except that additional glucose replaced the casein.

The results have been summarized in Table I. The decrease in the alcohol oxidation system as a result of protein depletion or inanition (1) was similar to the changes previously reported(2) for xanthine oxidase, and was somewhat greater than that found for many other liver enzymes(3).

Summary. 1. Weanling rat liver homogenate had about one-half the level of alcohol oxidation activity eventually achieved by adult rats on a chow diet (net oxygen uptake of 24 cmm per 10 minutes per 200 mg fresh liver for the adult normal rat). 85% or 75% respectively of this normal level of activity was reached by feeding chow or a purified 24% casein diet for 2 weeks. A protein-free diet removed about 85% of this enzymatic activity from the liver. 2. Adult rats responded similarly to these diets. Complete starvation for 7 days reduced this activity about 50%, while a 50% food restriction for 2 weeks gave less than a 20% decrease in the rate of alcohol oxidation.

1. Hegsted, D. M., Vitale, J. J., and DiGiorgio, J., *Fed. Proc.*, 1953, v12, 416.

2. Westerfeld, W. W., and Richert, D. A., *J. Biol. Chem.*, 1951, v192, 35.

3. Meikleham, V., Wells, I. C., Richert, D. A., and Westerfeld, W. W., *J. Biol. Chem.*, 1951, v192, 651.

Received June 10, 1953. P.S.E.B.M., 1953, v83.

Occurrence of Hexose-6-phosphate Dehydrogenase in Chorioallantoic Membrane of the Chick Embryo.* (20407)

ERNEST KUN.

From the Department of Medicine, Division of Infectious Disease, and Department of Biochemistry, Tulane University, New Orleans, La.

Previous experiments on the *in vitro* metabolism of excised chorioallantoic membranes of chick embryos dealt with changes in aerobic glycolysis which occurred following inoculation with viruses(1). In connection with this problem it became necessary to investigate the metabolic pathways of this tissue in more detail. The investigation reported here was undertaken as a part of these studies, and showed that the chorioallantoic membrane contains, besides the Embden-Meyerhof-Warburg type of glycolytic enzyme system(1), a glucose-6-phosphate oxidase.

Materials and methods. The embryonated eggs of white Leghorn and Rhode Island Red hens were obtained from a local hatchery. The age of the eggs varied from 6 to 13 days. The membranes were carefully excised and thoroughly washed in ice-chilled homogenizing medium which consisted of 0.15 *M* KCl containing 0.05% ethylene-diamine tetraacetic acid. The pH of the KCl solution was brought to 7.1 with KOH, and then 2 ml of 0.5 *M* glycylglycine buffer (pH 7.3) per 500 ml were added. The membranes of 5 to 8 eggs were combined and quickly ground with ice-cold (4°C) KCl solution in a glass homogenizer. The homogenate was kept in an ice bath prior to enzyme assays. The protein content of the homogenate was determined colorimetrically according to Lowry *et al.*(2), with crystalline bovine albumen as reference standard. Triphosphopyridine nucleotide (TPN, 60% pure), diphosphopyridine nucleotide (DPN, 90% pure), and glucose-1-PO_4 K-salt were obtained from Sigma Chemical Company, Inc. The Ba-salt of glucose-6-phosphate was a purified sample received through the courtesy of Dr. D. Broida (Sigma Chemical Co., Lot 91-20) Fructose-6-phosphate (purchased as Ba-salt from Nutritional Biochemicals Corp.) and fructose-1-6-diphosphate (HDP, Ca-salt, Sigma) were purified by ion exchange(3). Both hexose monophosphates and HDP were made up to 0.1 *M* solutions as K-salts. Pyocyanine was recrystallized twice from hot water after chloroform extraction of a commercial sample (Delta Chemicals), as described by Wrede and Starck(4). Janus green B and methylene blue were commercial samples. Isatin-6-

---

* This investigation was supported by a research grant from the National Microbiological Institute of the National Institutes of Health, Public Health Service.

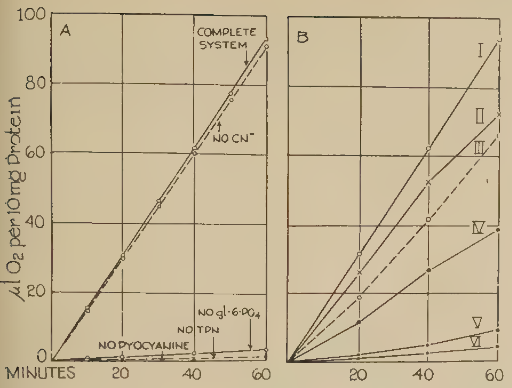


FIG. 1. Each vessel contains 1 ml homogenate. The following components are in the main compartment: A. Complete system: 10^{-2} *M* potassium glucose-6-phosphate, 10^{-2} *M* KCN(6), 1 μM TPN (dissolved in 0.2 ml 0.5 *M* glycylglycine buffer), and glass-distilled water to 2.6 ml. B. I. Complete system, containing no CN^-; II. Same as I, except with potassium iodoacetate in final concentration of 10^{-3} *M*; III. Glucose-6-phosphate replaced by equimolar fructose-6-phosphate; IV. Pyocyanine replaced by equimolar methylene blue; V., by Janus green B, and VI., by isatin-6-carboxylic acid (K-salt). The electron carriers are added as aqueous solutions in 0.3 ml volume, equivalent to 1 μM of each. 0.1 ml 20% KOH is placed in the center well with a strip of filter paper. Temp. 37°C; gas phase is air.

carboxylic acid was synthesized from m-aminobenzoic acid(5) and the final product after 2 recrystallizations made up to a 0.01 *M* solution as K-salt. Enzyme assays were carried out by conventional manometric technics. When CN^- was used in the presence of KOH in the center well the procedure of Krebs(6) was followed. The reduction of TPN was determined on the Beckman DU spectrophotometer at 3400 Å.

Results and discussion. The oxidation of hexose-6-phosphate by homogenates of chorioallantoic membranes was measured in a manometric test system. The effects of the components of the test system on the rate of O_2 absorption are summarized in Fig. 1 (A and B). The homogenizing medium as described above was chosen because in this salt solution enzyme activity is preserved for several hours. This stabilizing effect is due to the removal of traces of heavy metal impurities by the chelating agents employed. This observation is in agreement with the findings of von Euler and Adler(7), who reported enzyme inhibition by copper ions.

The use of CN^- in the manometric assay of "Zwischenferment" was described by Negelein and Gerischer(8); therefore it was tested in our system. As shown in Fig. 1A, CN^- has no appreciable effect on the rate of reaction, and it was omitted in further experiments. The pH optimum of the hexose-6-phosphate oxidase activity of whole homogenates, as measured in our test system, lies between 7.0 and 7.4. The final pH of the contents of respirometer flasks was 7.2 in all experiments. The rate of O_2 absorption by the complete test system was linear over a period of 60-90 minutes and proportional to the amount of homogenate. The rate of reaction is identical in air and 100% O_2 atmosphere. No reaction occurs if TPN, glucose-6-phosphate or pyocyanine are omitted. Similarly, if glucose, HDP, or glucose-1-phosphate is substituted for glucose-6-phosphate, or DPN for TPN, no O_2 uptake occurs in this test system (Fig. 1A). Fructose-6-phosphate as substrate maintains a steady rate of O_2 consumption which is, however, lower than that with glucose-6-phosphate (Fig. 1B). Iodoacetate in concentrations which strongly inhibit the triose phosphate dehydrogenase of the chorioallantoic membrane (10^{-4} *M* iodoacetate inhibits 90%) does not alter glucose-6-phosphate oxidation, and only higher concentrations (10^{-3} *M*) produce some inhibition.

As mentioned above, no O_2 uptake occurs in the absence of pyocyanine, which was found to be the most suitable carrier. Methylene blue, Janus green B, and isatin-6-carboxylic acid are increasingly poorer catalysts (Fig. 1B). Langenbeck, Weschky, and Gödde(9) found that carboxyisatins act as potent catalysts in the nonenzymatic dehydrogenation of alanine. A study on the interaction of isatin-6-carboxylic acid with other enzyme systems, besides hexose-6-phosphate dehydrogenase, is now being pursued.

The glucose-6-phosphate oxidase activity of homogenates of chorioallantoic membranes of chick embryos between the age of 6 to 13 days remains fairly constant, Qo^2 prot. (μl O_2 per 1 mg protein per 1 hour) ranging between 7.2-9.4 (11 enzyme assays). It was found previously that the reduction of certain dyes by dehydrogenases requires a flavoprotein present in mitochondria(10). It seemed of

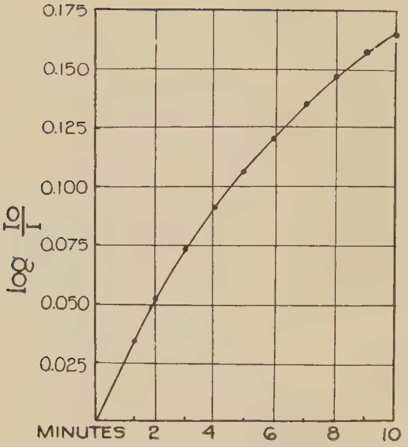


FIG. 2. The test system contains 0.3 ml centrifuged extract of chorioallantoic membrane (2.25 mg protein), 20 μM glucose-6-phosphate (0.2 ml), 1 μM TPN (in 0.2 ml glycylglycine buffer), and distilled water to a final volume of 3 ml. The reaction is started by the addition of either the enzyme, substrate, or coenzyme. Changes in optical density are recorded at 3400 Å in 1 cm cells. Temp. 24°C.

interest, therefore, to ascertain whether or not the electron transfer to O_2 occurs by means of a similar mechanism in the glucose-6-phosphate oxidase system of the chorioallantoic membrane. Homogenates were centrifuged at 25,000 x g for 60 minutes at 4°C and the sediment and clear supernatant fluid were separated. The sediment was resuspended in a volume of KCl solution equal to the original homogenate. Enzyme and protein assays were carried out in the whole homogenate, on the centrifugal supernatant fluid, and on the resuspended sediment. The following results were obtained: the total enzyme content, as expressed in μl O_2 absorbed by 1 ml of enzyme per hour, was 148.6 μl for the whole homogenate, 144.3 μl for the centrifugal supernatant fluid, and 4.2 μl for the particulate fraction. The protein content of the whole homogenate was 19.5 mg/ml, that of the supernatant fluid 12.5 mg/ml, and that of the resuspended particulate fraction 7.0 mg/ml. It is apparent that the enzyme activity of the homogenate is almost completely (96.5%) recovered in the supernatant fluid, indicating that enzyme systems of the particulate fraction do not participate in this reaction. The rate of reduction of TPN by the centrifugal supernatant fluid is determined spectrophotometrically at room temperature (Fig. 2). The rate of reduction of TPN decreases with time, an observation which can be explained on the basis that reduced TPN competes with oxidized TPN for the dehydrogenase(12). The reduction of dichlorophenolindophenol in the test system of Haas(11) can also be successfully employed if the centrifuged extracts are first dialyzed in order to minimize endogenous dye reduction.

Conclusions. It is evident that the chorioallantoic membranes of chick embryos can oxidize glucose-6-phosphate by an enzyme system similar to that discovered by Warburg and Christian(12,13). The centrifugal supernatant fluid of homogenates contains the complete catalytic system necessary for oxidation of glucose-6-phosphate as measured in our test system. The reaction is completely dependent on hexose-6-phosphate and TPN. This finding is of particular interest since it indicates that the chorioallantoic membrane contains an alternative pathway of glucose metabolism besides the glycolytic system described previously(1).

Summary. Homogenates of chorioallantoic membranes of the chick embryo oxidize glucose-6-phosphate or fructose-6-phosphate aerobically if TPN and pyocyanine are present. The enzyme activity which is recovered in the centrifugal supernatant fluid does not change appreciably with age of the embryo between 6 and 13 days.

The author is indebted to Drs. M. H. D. Smith, E. D. Kilbourne, and I. E. Tateno for their helpful cooperation in obtaining the chorioallantoic membranes. The gift of ethylene-diamine tetraacetic acid (Sequestrene) from Alrose Chemical Co. is acknowledged with thanks.

---

1. Kun, E., and Smith, M. H. D., Proc. Soc. Exp. Biol. and Med., 1950, v73, 628.

2. Lowry, O. H., Rosebrough, N. J., Farr, A. L, and Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.

3. McCready, R. M., and Hassid, W. Z., *J. Am. Chem. Soc.*, 1944, v66, 561.

4. Wrede, F., and Starck, E., *Z. physiol. Chem.*, 1929, v181, 58.

5. Waldmann, H., *J. prakt. Chem.*, 1937, v147, 338.

6. Krebs, H. A., *Biochem. J.*, 1935, v29, 1620.

7. von Euler, H , and Adler, E., *Z. physiol. Chem.*, 1935, v232, 10.

8. Negelein, E., and Gerischer, W., *Biochem. Z.*, 1936, v284, 289.
9. Langenbeck, W., Weschky, L., and Godde, O., *Ber.*, 1937, v70B, 672.
10. Kun, E., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 195.
11. Haas, E., *J. Biol. Chem.*, 1944, v155, 333.
12. Warburg, O., and Christian, W., *Biochem. Z.*, 1932, v254, 438.
13. ———, *Biochem. Z.*, 1931, v238, 131.

Received June 10, 1953. P.S.E.B.M., 1953, v83.

Chromatography of α-ϵ-Diaminopimelic Acid on Starch Columns.* (20408)

EVA SOTO FIGUEROA† AND FLORENCE B. SEIBERT.
From the Henry Phipps Institute, University of Pennsylvania, Philadelphia, Pa.

In connection with studies on the amino acid composition of certain proteins it was desirable to know the behavior on starch chromatographic columns of the recently discovered amino acid, α-ϵ-diaminopimelic acid‡ (called in this paper DAP) isolated by Work (1,2). It was of interest also to note its mobility by means of paper electrophoresis.

Procedure. The starch columns (0.9 x 30 cm) were prepared as recommended by Moore and Stein(3), and the effluent values of the DAP and certain amino acids added to the solution as markers were followed by means of the ninhydrin reaction on fractions collected by an automatic fraction collector and drop-counting apparatus(4). Two solvents were used in this study, 1:2:1 (n-butyl-n-propyl, 0.1 N-HCl) and 2:1(n-propyl-0.5N HCl). The DAP was dissolved in 0.1 N-HCl to which was then added 4 volumes of 1:2:1 solvent, giving a final concentration of 3 mg DAP per ml. Proper aliquots were added to the columns to give the quantities designated in Table I. For paper electrophoresis§ the different mixtures of amino acids as used in the chromatography experiments were placed on strips of Whatman No. 1 filter paper and run at 130 volts and 25 milliamperes for 2 hours in barbiturate buffer pH 8.6. The buffer was made as follows. Sodium barbiturate, 29.428 g, sodium acetate 3 H_2O, 19.428 g and N/10 HCl 180 ml were dissolved in 3000 ml distilled water and then diluted with an equal amount of distilled water. The same amino acids as mentioned above were also dissolved directly in the buffer before placing them on paper strips. At the end of the experiment the strips were dried in an oven, sprayed with ninhydrin and heated 4 minutes at 90°C.

Results. In the first chromatographic experiment leucine and DAP were put on the column with the 2:1 solvent and the DAP was found to emerge at an effluent value of 80 ml, as shown in Table I. It remained on the column in experiment 2 when the 1:2:1 solvent was used and emerged only after the solvent was replaced with 2:1 solvent and then at an effluent value about 65 ml past this change. These values were suggestive of the position for lysine. Therefore, in a third experiment lysine was added to DAP and the mixture placed on a column. At the same time a mixture of proline and DAP was run in tandem. As shown in Table I, DAP emerged identically with lysine as a single sharp peak and the recovery was close to the theoretical value. (Fig. 1).

---

* Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and by a grant from the Committee on Medical Research of the American Trudeau Society, Medical section of the National Tuberculosis Association.

† Fellow of the International Branch of the United States Public Health Service.

‡ For these studies some of the amino acid was generously given to us by Dr. Work.

§ Paper strip model electrophoresis apparatus obtained from Arthur H. Thomas Co., Philadelphia, Pa.

TABLE I. Effluent Values and Recovery of α-ϵ-Diaminopimelic Acid on Starch Columns. 4 experiments.

Solvents used	Changed solvent at effluent value, ml	Amino acid	Effluent value, ml	mg amino acid Added	mg amino acid Recovered
2:1		Leucine	10	.3	.25
		+ DAP	80	.5	.55
1:2:1 & 2:1	104.4	Leucine	14	.3	.31
		+ DAP	169	.5	.55
1:2:1 & 2:1	101.4	Lysine	171	.3	.59
		+ DAP	171	.3	
1:2:1 & 2:1	99.5	Proline	62	.3	.31
		+ DAP	173	.3	.32

When studied by means of paper electrophoresis at 25 milliamperes for 2 hours in barbiturate buffer, pH 8.8, as described above, the DAP was found to migrate only slightly slower 42 mm, than proline 45 mm, considerably slower than lysine 80 mm, and faster than glutamic acid 0 mm. See Fig. 2. These relative migrations were found to be the same whether the amino acids were dissolved in the 1:2:1 solvent or in the buffer solution (pH 8.6) used for the paper electrophoresis.

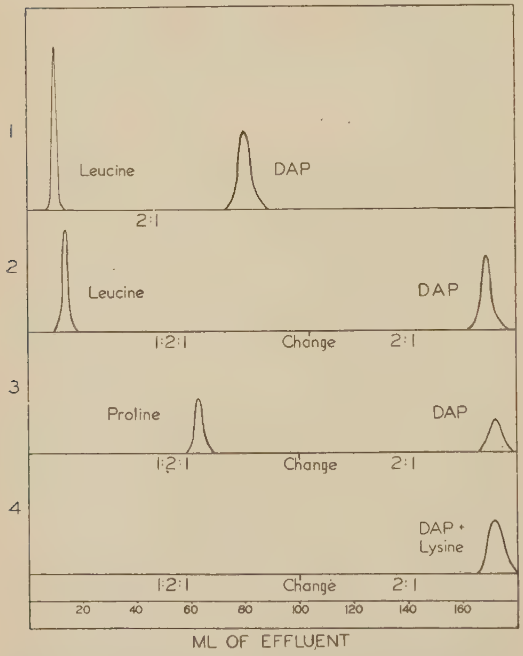


FIG. 1. Chromatographic diagrams on starch columns (0.9 × 30 cm) of DAP mixed with leucine (1 and 2), with proline (3), and with lysine (4). Solvents were 1:2:1 n-butyl-n-propyl –0.1 N-HCl and 2:1 n-propyl –0.5 N-HCl.

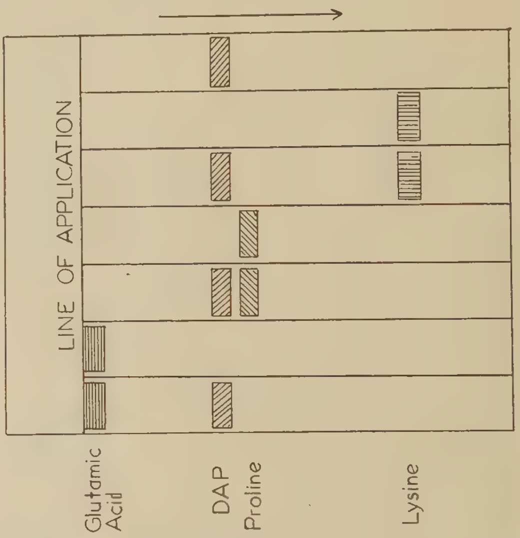


FIG. 2. Paper electrophoretic diagrams of DAP and DAP mixed with glutamic acid, proline or lysine. Barbiturate buffer, pH 8.6, for 2 hr at 25 milliamperes.

Discussion. The fact that the DAP is eluted from starch at the same effluent value as lysine is of interest in view of the suggestion of Work(5) that this amino acid may be a biological precursor of lysine and also of the finding(1) that the R_F of the DAP was similar to that of lysine by paper chromatography in collidine. In phenol, on the other hand, she found DAP and glutamic acid to travel more closely.

These chromatographic absorption properties by starch or by paper are obviously not dependent upon electrical charge, since DAP does not migrate in electrophoresis like either lysine or glutamic acid. Instead, its migration is similar to that of the neutral amino acid proline, whose isoelectric point is pH 6.3, *i.e.*, quite close to that of DAP-pH 5.8(6). In fact, Work(2) has isolated it with the neutral amino acids by electrodialysis.

Summary. The new amino acid, discovered by Work, α-ε-diaminopimelic acid, is absorbed on and eluted from starch similarly to lysine by the solvents 1:2:1 n-butyl-n-propyl-0.1 N-HCl and 2:1 n-propyl-0.5 N-HCl. It migrates in electrophoresis in barbiturate buffer pH 8.6 between glutamic acid and lysine but close to proline, whose isoelectric point is near to that of the diaminopimelic acid.

---

1. Work, E., *Nature*, 1950, v165, 74.
2. ———, *Biochem. J.*, 1951, v49, 17.
3. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1949, v53, 178.
4. Stein, W. H., and Moore, S., *J. Biol. Chem.*, 1948, v176, 337.
5. Dewey, D. L., and Work, E., *Nature*, 1952, v169, 533.
6. Work, E., 11° Congres International De Biochimie, Paris, 20-27, Juillet, 1952, page 98.

Received June 11, 1953. P.S.E.B.M., 1953, v83.

Effect of Adrenal Cortical Extract upon Work Output and Glucose Tolerance of Adrenalectomized-Eviscerate Rat. (20409)

Dwight J. Ingle, James E. Nezamis, and Robert C. Meeks.
From the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

The data of this study confirm an earlier report(1) that adrenal cortical extract (ACE) has a positive effect upon the work output of adrenalectomized-eviscerate rats. There is an accompanying decrease in the tolerance for intravenously administered glucose.

Methods. Male rats of the Sprague-Dawley strain from the Upjohn breeding colony were maintained on a diet of Archer Dog Pellets. The technic of evisceration, which is done in 2 stages, has been described(2). When the animals reached 250 ± 2 g they were anesthetized (intraperitoneal injection of 18 mg of cyclopal sodium) and were subjected to the second stage of the operation in which the adrenal glands were removed with the other intra-abdominal organs. The stimulation of muscle was started immediately following evisceration. The procedure was according to Ingle(3) with the following modifications. A Nerve Stimulator, Model B, Upjohn, was used to stimulate muscle 5 times per second by a D.C. pulse of 20 ma having a duration of 20 msec. The gastrocnemius muscle of the left hind leg was weighted with 100 g. In Exp. 1 and 2 this leg only was stimulated. In Exp. 3, both back legs were stimulated. The distance that the weight was lifted was registered on automatic work recorders. Each recorder revolution represented approximately 400 g-cm of work. A solution containing glucose, regular insulin (Lilly), penicillin and streptomycin with and without ACE [1 glycogen unit(4) per ml] was infused into the jugular vein of each rat. The animals were enclosed in a cabinet with temperature constant at 28 ± 0.5 C. The infusions were started simultaneously with the stimulation of muscle immediately following the operation. The analyses of glucose were made on tail blood by the method of Miller and Van Slyke (5).

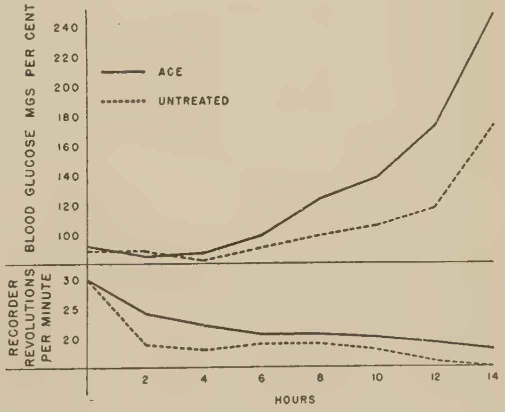


FIG. 1. Effect of ACE upon work output and glucose tolerance of adrenalectomized-eviscerate rats given a fluid load of 40 ml per rat per 24 hr. Stimulate one back leg. Averages.

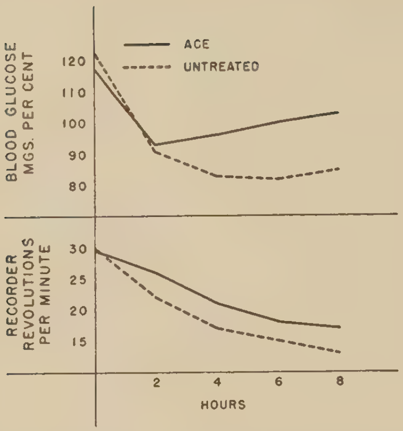


FIG. 2. Effect of ACE upon work output and glucose tolerance of adrenalectomized-eviscerate rats given a fluid load of 20 ml per rat per 24 hr. Stimulate one back leg. Averages.

Experiments and results. In experiment one, 31 adrenalectomized-eviscerate rats were each given 40 ml of solution per 24 hours containing 4 units of insulin, 10,000 units of penicillin, 20 mg of streptomycin and a glucose load of 125 mg per 100 g of rat per hour. Fifteen of the rats each received 20 glycogen units of ACE per 24 hours added to the above solution. The remaining 16 rats did not receive ACE. The level of blood glucose was determined at 2-hour intervals during the first 14 hours. The data of Fig. 1 show that the work output of the rats treated with ACE was enhanced above that of the untreated rats and that there was an accompanying elevation in blood glucose. At the end of 24 hours the average total number of recorder revolutions for the rats treated with ACE was 26429 ± 1470 and the corresponding value for the untreated rats was 21756 ± 1047. The difference (4673 ± 1805) meets the usual requirements for statistical reliability.

In Exp. 2, 35 adrenalectomized-eviscerate rats were each given 20 ml of solution per 24 hours containing the same loads of glucose, insulin and antibiotics as in Exp. 1. Seventeen of the rats each received 12 glycogen units of ACE per 24 hours added to the above solution. The remaining 18 rats did not receive ACE. The level of blood glucose was determined at 2-hour intervals during the first 8 hours. The data of Fig. 2 show that the work output of the rats treated with ACE was greater than that of the untreated rats and that there was an accompanying elevation in blood glucose. At the end of 24 hours the average total number of recorder revolutions for the rats treated with ACE was 20364 ± 997 and the corresponding value for the untreated rats was 14451 ± 866. The difference (5913 ± 1320) meets the usual requirements for statistical reliability.

In Exp. 3, 36 adrenalectomized-eviscerate rats were each given 20 ml of solution per 24 hours representing a glucose load of 200 mg per 100 g of rat per hour and the same loads of insulin and antibiotics used in Exp. 1 and 2. Eighteen rats received 12 glycogen units of ACE per 24 hours added to the above solution and the remaining 18 rats did not receive ACE. In this experiment both back legs were stimulated. The level of blood glucose was determined at 2-hour intervals during the first 8 hours. The data of Fig. 3 show that the work output of the group treated with ACE was greater than that of the untreated group. By the 6th hour, muscular responsiveness was lost in all of the animals of the un-

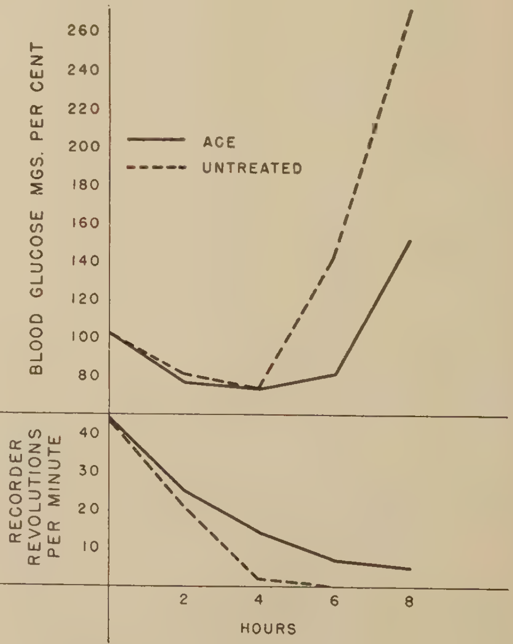


FIG. 3. Effect of ACE upon work output and glucose tolerance of adrenalectomized-eviscerate rats given a fluid load of 20 ml per rat per 24 hr. Stimulate both back legs. Averages.

treated group whereupon the level of blood glucose rose sharply above the values for the treated rats which continued to work for periods up to 12 hours. The total average number of recorder revolutions for the group treated with ACE was 9482 ± 613 and the corresponding value for the untreated group was 5361 ± 261. The difference (4121 ± 1345) meets the usual requirements for statistical reliability.

Discussion. The presence of the liver and other intra-abdominal organs is not required for an effect of adrenal cortical hormones upon muscle work. This does not mean that the normal role of the liver in adrenal cortical physiology is unimportant.

The stimulation of muscle in the rat greatly accelerates the oxidation of glucose(6). On this basis it would be expected that the greater work output of the rats treated with ACE would increase glucose tolerance above that of untreated rats. Although muscle work does have a major effect upon glucose tolerance as is evidenced by the results of experiment 3 (Fig. 3), the effect of ACE in decreasing glucose tolerance can mask the effect of small differences in work output as shown in experiments 1 and 2 (Fig. 1 and 2). This effect of adrenal cortical extract in suppressing the glucose tolerance of eviscerated rats was previously observed in non-working animals(7). The basis of this extra-hepatic effect of ACE upon the utilization of glucose is not understood.

Summary. The continuous intravenous injection of adrenal cortical extract to adrenalectomized-eviscerate rats improved work output during the faradic stimulation of muscle in one hind leg. There was a concomitant elevation of blood glucose above the level of the untreated rats when the animals continued to work. When both hind legs were stimulated, muscular responsiveness was completely lost in each of the untreated rats within 6 hours and there was an immediate rise in the level of blood glucose above the values for the treated rats which continued to work.

1. Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1938, v39, 151.

2. ———, *Exp. Med. and Surg.*, 1949, v7, 34.

3. ———, *Endocrinol.*, 1944, v34, 191.

4. Pabst, M. L., Sheppard, R., and Kuizenga, M. H, *Endocrinol.*, 1947, v41, 55.

5. Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, v114, 583.

6. Stetten, DeWitt, Jr., Morley, E. H., and Ingle, D. J., Unpublished data.

7. Ingle, D. J., Prestrud, M. C., Nezamis, J. E., and Kuizenga, M. H., *Am. J. Physiol.*, 1947, v150, 423.

Received June 11, 1953. P.S.E.B.M., 1953, v83.

Soyin, A Toxic Protein from Soybean III. Immunochemical Properties.* (20410)

IRVIN E. LIENER AND JOSEPH E. ROSE. (Introduced by Paul D. Boyer)

From the Department of Agricultural Biochemistry, University of Minnesota, St. Paul, Minn.

Some of the biological and physical properties of soyin, a toxic protein recently isolated from defatted soybean flour, have been described previously(1-4). Electrophoretic and diffusion measurements of soyin† revealed a high degree of homogeneity, whereas sedimentation data indicated contamination with a small quantity of low molecular weight impurity(3). In the present investigation immunological technics were employed to obtain more precise information regarding the homogeneity of soyin. The data which were obtained also permitted a calculation of the soyin content of the original soybean flour from which the soyin had been prepared.

Methods. Preparation of soyin and crude

---

* Paper No. 3027 Scientific Journal Series, Minn. Agr. Exp. Station.

† The most highly purified fraction which has thus far been possible to prepare.

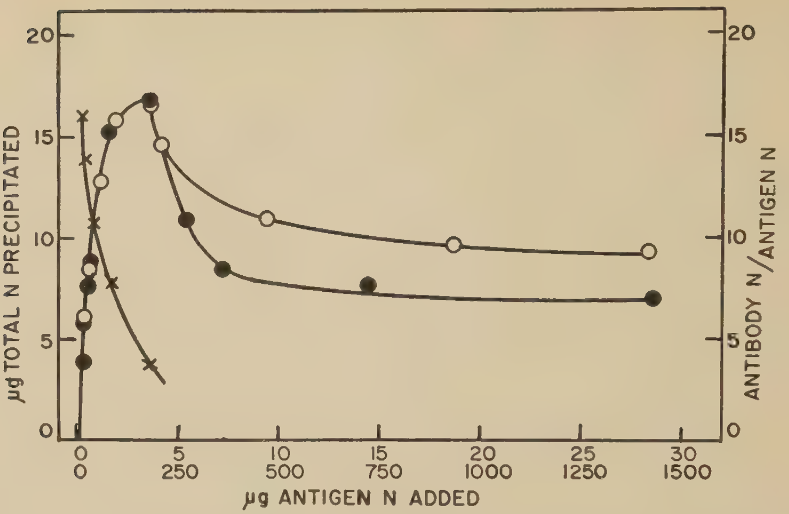


FIG. 1. Observed precipitin reaction of soyin 'Antiserum A' with soyin (-●—●-) and CSBE (-○—○-). Upper and lower abscissae refer to quantity of soyin N and CSBE N added respectively. Plot of the ratio of antibody N (total N precipitated minus soyin N) to antigen (soyin) N vs antigen (soyin) N is shown by the curve (-×—×-).

soybean extract (CSBE). The soyin preparation used in this work was the same as that used for the physical studies previously reported(3). The crude soybean extract was prepared by suspending 1 part of defatted soybean flour[‡] in 10 parts of 0.9% NaCl. The pH was adjusted to 6.7, and the solution was stirred vigorously for 1 hr at room temperature. The clear, yellow supernatant that remained after centrifuging will be hereafter referred to as CSBE. Hemagglutinating tests (described below) on extracts of the insoluble residue showed that all of the hemagglutinating activity had been removed by the first extraction. *Preparation of soyin antiserum.* A rabbit was immunized by injecting soyin intravenously at levels of 1.5 mg, 3.0 mg, and 6.0 mg 3 times per week during the first, second, and third weeks respectively. Five days after the last injection blood was withdrawn by cardiac puncture and allowed to clot. The serum, centrifuged free of the retracted clot, was preserved by adding 0.1 mg merthiolate per ml, and stored in the refrigerator. No attempt was made to remove the complement from the serum. Serum obtained from this rabbit will be referred to as 'Antiserum A'. *Precipitin technic.* Various dilutions of soyin or CSBE (0.9% NaCl as the diluent) in a volume of 1 ml were added to 1 ml of diluted soyin antiserum (1 part serum + 2 parts of 0.9% NaCl). Appropriate controls of diluent plus antiserum and diluent plus antigen were likewise prepared. The tubes were held at 37°C for 1 hr, and kept in the refrigerator for at least 48 hrs before centrifuging. The precipitates were washed according to the procedure of Kabat and Mayer(5) and analyzed for nitrogen by the method of Lanni *et al.*(6). The latter procedure was also found to be very satisfactory for determining the nitrogen content of CSBE and solutions of soyin. *Toxicity and hemagglutinating activity.* The methods used for determining toxicity and hemagglutinating activity have been described previously(1). The effect of soyin antiserum on the hemagglutinating activity of soyin and CSBE was assessed in a quantitative manner by the following modification of the procedure described by Kabat and Mayer(5). To various dilutions of the antiserum (with 0.9% NaCl as the diluent) in a volume of 0.5 ml was added a constant amount of soyin or CSBE in a volume of 0.2 ml. After the tubes were kept at 37°C for 1 hr, 0.2 ml of a 4% washed suspension of rabbit erythrocytes in 0.9%

[‡] Nutrisoy XXX, Archer-Daniels-Midland Co., Minneapolis, Minn.

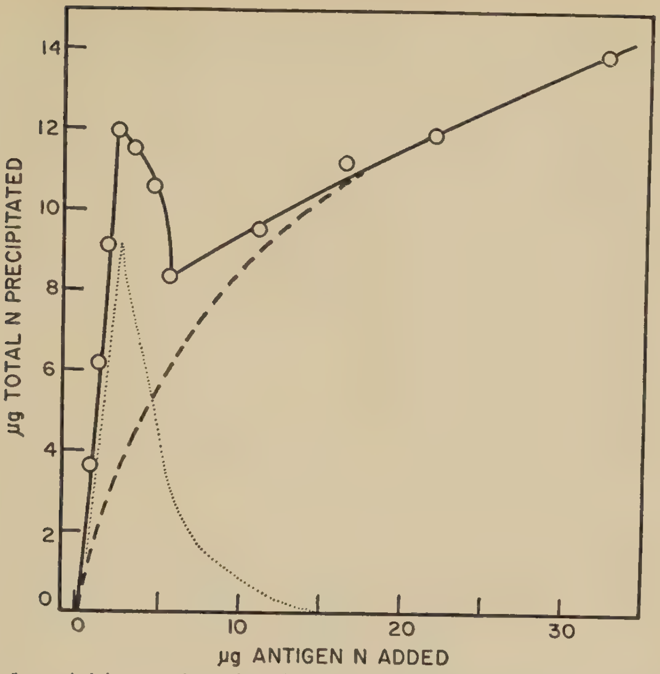


FIG. 2. Observed precipitin reaction of soyin antiserum B with soyin (–○——○–). Extrapolation to 0 of that portion of the observed curve extending beyond 15 μg antigen N added is shown by the curve (– – – – –). Subtraction of the extrapolated curve from the observed curve gives the true soyin-antisoyin curve (. . . . . . .).

NaCl was added to each tube. After holding at 37°C for an additional hr, the highest dilution of the serum causing complete inhibition of agglutination was recorded.

Results and discussion. Precipitin tests. In Fig. 1 are presented the data obtained when 'Antiserum A' was tested against soyin and CSBE. Plotting the total amount of N precipitated as a function of soyin N added gave a curve with the characteristic zones of antibody excess, equivalence, and antigen excess. The curve obtained with CSBE could be superimposed on the soyin curve in the region of antibody excess by reducing the units along the antigen axis by 1/50. If one assumes that these curves represent the reaction of a single antigen and a single antibody, the soyin content of CSBE would be 2%.§ Actually, however, the following lines of evidence suggested that the system involved here was not a simple reaction between 2 single components: a) as shown in Fig. 1 a plot of the ratio of antibody N (Ab) to antigen N (An) vs An failed to give a straight line such as would be expected from the Heidelberger-Kendall equation(7) Ab/An = a-bAn for a homogeneous antigen-antibody system, b) tests on the supernatants revealed an excess of both antigen and antibody at the point of maximum precipitation, and c) complete solubility of the specific precipitates in the region of antigen excess was not obtained. According to Cohen *et al.*(8) these facts provide strong evidence that the antibody-excess portion of the curves shown in Fig. 1 actually represents the sum of the reactions of soyin with its antibody and of a minor impurity with its antibody. This would, of course, invalidate any attempt to determine the soyin content of CSBE without first applying suitable corrections for the impurity.

To resolve the problem imposed by the presence of an impurity, advantage was taken of the fact that prolonged immunization favors antibody production against minor impurities (9). A rabbit injected with soyin according

---

§ The phrase "soyin content of CSBE" as employed in this paper refers to the ratio of soyin N to CSBE N (x 100 if expressed percentagewise).

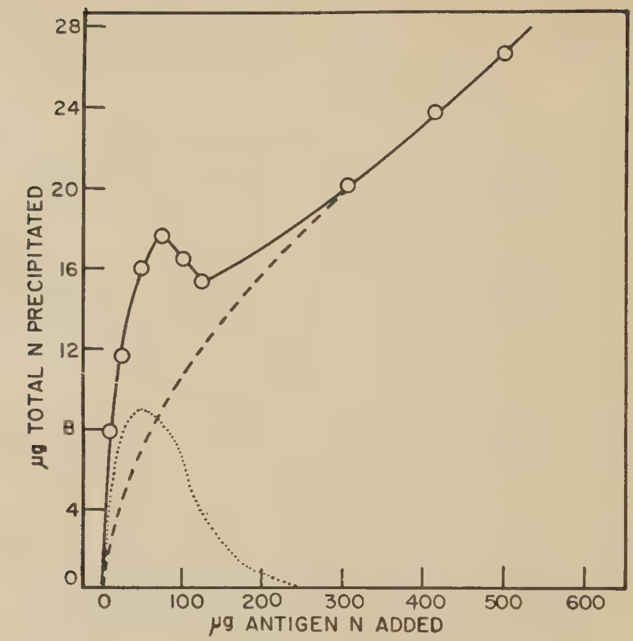


FIG. 3. Observed precipitin reaction of soyin antiserum B with CSBE (-○—○-). Extrapolation to 0 of that portion of the observed curve extending beyond 250 μg antigen N added is shown by the curve (– – – – –). Subtraction of the extrapolated curve from the observed curve gives the true soyin-antisoyin curve (.......).

to the previously described schedule was given additional injections of 2 mg at intervals of 1 week over a period of two months. As shown in Fig. 2 and 3 the serum from such an animal ('Antiserum B') when tested against soyin and CSBE now gave unequivocal evidence for the presence of at least 2 components.[‖] In fact the rabbit produced more antibody against the impurity than against soyin itself. Following the procedure suggested by Cohen *et al.*(8), the equations describing the reaction of the impurity with its antibody (portions of the curves exceeding 15 and 250 μg antigen N in Fig. 2 and 3 respectively) were calculated, and the curves were extrapolated to 0. The true soyin-antisoyin curve is then given by the difference between the observed and extrapolated portion of the curve. It can be readily shown that the true soyin-antisoyin curves obtained with soyin and CSBE (Fig. 2 and 3 respectively) are superimposable in the region of antibody excess when the ratio of soyin N to CSBE N along the antigen axis is 1:10. This would indicate therefore that the soyin content of CSBE is closer to 10% than the 2% value which had been obtained when the presence of the impurity had not been taken into account.

'Antiserum B' gave a negative capillary precipitin test with the crystalline soybean trypsin inhibitor described by Kunitz(11).[¶] This fact coupled with the observation that soyin has no antitryptic activity(1) makes it extremely unlikely that the impurity revealed by the quantitative precipitin test is the soybean trypsin inhibitor of Kunitz.

Toxicity and hemagglutinating data. A comparison of the toxicity and hemagglutinating activity of soyin and CSBE is made in Table I. The inhibitory effect of 'Antiserum A' on the hemagglutinating activity of soyin and CSBE is shown in Table II. The results obtained with 'Antiserum B' were essentially the same as those given for 'Antiserum A'. The soyin content of CSBE calculated from

---

[‖] The agar diffusion technic of Becker and Munoz (10) revealed presence of two distinct bands when 'Antiserum B' was reacted with soyin.

[¶] Purchased from Worthington Biochemical Laboratory, Freehold, N. J.

TABLE I. Toxicity and Hemagglutinating Activity of Soyin and a Crude Soybean Extract (CSBE).

	Soyin	CSBE
Toxicity[*]		
LD_{50}, mg N/kg body wt	7.0	77.0
% toxic N	100.0[†]	9.1
Hemagglutinating activity		
N content of original sol., μg N/ml	57.5	300.0
Highest dilution showing agglutination	1:16	1:8
Hemagglutinating titer, μg N[‡]	3.6	37.5
% hemagglutinin N	100.0[†]	9.6

[*] Administered intraper. into young weanling rats as described in(1).

[†] Assumed as point of reference.

[‡] N content of highest dilution showing positive evidence of agglutination under conditions specified in(1).

TABLE II. Inhibition of Hemagglutinating Activity of Soyin and a Crude Soybean Extract (CSBE) by Soyin 'Antiserum A.'

	Soyin	CSBE
μg N added to each tube	11.5	60.0
Dilution of antiserum causing complete inhibition of agglutination	1:4	1:8
μg N neutralized by 1 ml antiserum	46	480
% hemagglutinin N	100.0[*]	9.6

[*] Assumed as point of reference.

TABLE III. Calculation of Soyin Content of Defatted Soybean Flour.

(a) mg CSBE N/g flour[*]	49.5
(b) mg soyin N/mg CSBE N[†]	.095
(c) Total mg soyin N/g flour (a × b)	4.7
(d) mg soyin N/g soyin[‡]	146.0
(e) % soyin in flour (c/d × 100)	3.2

[*] This represents total amount of N extracted from 1 g soybean flour by 10 ml 0.9% NaCl at pH 6.7 and 25°C.

[†] Avg value for soyin content of CSBE based on quantitative precipitin, toxicity, and hemagglutinating data. See Tables I and II in conjunction with text.

[‡] N content of soyin on dry wt basis = 14.6%.

toxicity data, hemagglutinating activity and inhibition of hemagglutinating activity by soyin antiserum was 9.1%, 9.6%, and 9.6% respectively. These values are in substantial agreement with the amount of immunologically active soyin present in CSBE (10.0%).

Soyin content of soybean flour. Using an average value of 9.5% for the soyin content of CSBE, it is possible to calculate the soyin content of the soybean flour from which soyin was prepared. As shown in Table III this value is approximately 3%. This information proved to be particularly useful in rat growth studies designed to determine to what extent soyin was responsible for the poor nutritive value of raw soybean protein(2). In these experiments it was necessary to incorporate soyin into a basal ration containing heated soybean flour at a level which approximated the soyin content of a similar ration containing the unheated flour. These studies showed that soyin accounted for one-half of the growth inhibition that is obtained when unheated soy flour replaces the properly heated flour in the diet.

Miscellaneous observations. Anaphylaxis. Guinea pigs were sensitized with an intramuscular injection of 0.1 mg soyin, and, three weeks later, were challenged with an intracardial dose of 20 mg. Death which ensued within 20 minutes was preceded by symptoms characteristic of anaphylactic shock (rubbing of the nose, dyspnea, and convulsions). Nonsensitized controls receiving the same intracardial dose of soyin also succumbed, but death in this instance was delayed several hours with no evidence of anaphylaxis.

An attempt to produce active immunization. Sera obtained from rats which had been given intramuscular injections of soyin (0.1 mg 3 times per week for 3 weeks) gave a positive capillary precipitin test with soyin. Such animals, however, were just as susceptible to the toxic effects of soyin as normal controls.

Effects of oral ingestion. Experiments were conducted to ascertain if soyin is absorbed from the gastrointestinal tract of animals consuming the unheated flour. Sera taken from rats fed diets containing unheated soybean flour gave no precipitin reaction with soyin. Guinea pigs fed diets containing the raw flour did not become sensitized to a subsequent shocking dose of soyin, nor did sensitized guinea pigs display any evidence of anaphylactic shock when they were subsequently fed diets containing raw soy flour. Soyin, administered to rats by stomach tube up to a level of 500 mg/kg, did not produce death. This is about 10 times the dose which is toxic intraperitoneally. This evidence strongly suggests that soyin may not be absorbed, or it may be inactivated as the result of digestive processes *in vivo*. This view is not necessarily inconsistent with the previous observation that the growth inhibition of rats fed soyin is simply the result of lowered food intake(2).

Summary. The quantitative precipitin technic revealed that the most highly purified preparation of soyin available was not strictly homogeneous as had been previously indicated by electrophoretic and diffusion measurements. By applying suitable corrections for the minor impurity present in soyin, the soyin content of a crude saline extract of defatted soybean flour was found to be approximately 10%. Toxicity and hemagglutinating data gave results which were in substantial agreement with this value. The soyin content of defatted soybean flour is about 3%.

---

1. Liener, I. E., and Pallansch, M. J., *J. Biol. Chem.*, 1952, v197, 29.

2. ———, *J. Nutrition*, 1953, v49, 527.

3. Pallansch, M. J., and Liener, I. E., *Arch. Biochem. and Biophys.*, in press.

4. Liener, I. E., Pallansch, M. J., and Rose, J. E., *Fed. Proc.*, 1953, v12, 790.

5. Kabat, E. A., and Mayer, M., *Experimental Immunochemistry*, Charles C. Thomas, Springfield, 1948.

6. Lanni, F., Dillon, M. L., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 4.

7. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, v62, 697.

8. Cohen, M., Wetter, L. R., and Deutsch, H. F., *J. Immunol.*, 1949, v61, 283.

9. Cohen, M., *Methods in Medical Research*, The Year Book Publishers, Inc., Chicago, 1952, v5, 280.

10. Becker, E., and Munoz, J., *J. Immunol.*, 1950, v65, 47.

11. Kunitz, M., *Science*, 1945, v101, 668.

Received June 15, 1953. P.S.E.B.M., 1953, v83.

Excretion of 3-(3-Amino-2,4,6-Triiodophenyl)-2-Ethyl-Propanoic Acid (Telepaque) by Man.* (20411)

J. SPALDING SCHRODER AND DONALD ROONEY. (Introduced by Arthur P. Richardson.)

From the Department of Medicine, Emory University School of Medicine, Emory University, Ga.

Recent reports(1-8) conclude that 3-(3-amino-2,4,6-triiodophenyl)-2-ethyl-propanoic acid (Telepaque) is superior to any of the cholecystographic media now available because of a significant reduction in the incidence of undesirable side effects and better gallbladder visualization. Studies on the excretion of this material by man have not been reported. McChesney and Wyzan(9) have studied the excretion of Telepaque by rats and recovered from 77.5% to 84.5% of ingested dye in the feces and from 2.1% to 4.8% in the urine, after the animals had been on the Telepaque-containing diet for 7 days. 11.3% to 11.8% was recovered from the digestive tract, and most of this material would have appeared in the feces in time. These findings are in sharp contrast to the excretion of beta-(4-hydroxy-3,5-diiodophenyl)-alpha-phenyl-propionic acid (Priodax) by man as reported by Crismer(10), who was able to recover 19% of the total ingested Priodax from the feces and 74% from the urine. The purpose of this communication is to report our findings on the excretion of Telepaque by man.

Method. Two grams of Telepaque (containing 1333.6 mg iodine) was given to five adults with normal gastrointestinal tracts. The 4 tablets were taken with supper at 6 P.M. when collection of 24-hour urine and fecal specimens was started. All the urine and feces were collected, thoroughly homogenized, and analyzed for total iodine present during the five 24-hour periods following the administration of the Telepaque. The iodine

---

* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

TABLE I. Iodine Recovery, Following Oral Ingestion of 1333.6 mg Iodine (2 g of Telepaque). 5 subjects.

	Urine		Feces		
Day	mg I_2 recovered	% of total dose	mg I_2 recovered	% of total dose	Total % recovered
1	110.2		388		
2	50.2		425		
3	23.1		147		
4	4.7		34.2		
5	0		19.6		
6	0		9.8		
7	0		4.3		
	188.2	14.2	1027.9	77.0	91.2
1	98.5		594		
2	57.8		216.5		
3	24.8		79.4		
4	0.6		36.6		
5	0		6.1		
	181.7	13.6	932.6	70	83.6
1	76.2		472.8		
2	37.4		392.4		
3	12.6		158.5		
4	5.6		60.7		
5	0		3.8		
	131.8	9.9	1088.2	81.7	91.6
1	86.3		362		
2	54.0		349		
3	39.6		126		
	179.9	13.5	837	62.7	76.2
1	82.6		731		
2	33.5		238		
3	27.8		26.4		
4	3.1		28		
5	0		8.9		
	147.0	11	1032.3	77.5	88.5

determinations were made by one of us (D.R.) according to the method described by Bang and Georg(11) with some modifications. Bang and Georg devised and used this method for a study of iodophthalein in various body fluids with an error of determination of 0.5 to 1 μg using 0.0004715 N thiosulfate for the titration. In our study with Telepaque, 0.001 N thiosulfate was used to facilitate titration with a 2.0 ml self-filling burette and because larger concentrations of iodine were present. Since the thiosulfate in the Telepaque study was about twice as strong and the samples a little smaller, an error of 2 to 5 μg may be expected per titration, which would affect the results very little.

Results. The iodine analyses of the samples and the percent of iodine recovered are presented in Table I.

Summary and conclusions. This study shows that 86% of the Telepaque was eliminated in 5 days, 12.4% by way of the urine, and 73.6% by way of the feces, in human subjects. Fourteen per cent was not accounted for, although the most probable route is via the feces in smaller and smaller amounts beyond the 5-day period of study. A higher percentage of Telepaque was thus recovered from the urine than the 2.1% to 4.8% previously reported(9) from the urine of experimental animals. The higher fecal and lower urinary excretion of Telepaque, in contrast to the 19% fecal and 74% urinary excretion of Priodax reported by Crismer, may be related to the superiority of the former as a cholecystographic medium.

The authors wish to acknowledge the encouragement and suggestions of Dr. H. S. Weens, Chairman of the Department of Radiology, Emory University

School of Medicine.

1. Archer, S., Hoppe, J. O., and Lewis, T. R., *J. Am. Pharm. Assn.*, 1951, v40, 143, 617.
2. Hoppe, J. O., and Archer, S., *Fed. Proc.*, 1951, v10, 975.
3. ———, *Fed. Proc.*, 1949, v8, 303.
4. Morgan, R. H., and Stewart, H. B., *Radiol.*, 1952, v58, 231.
5. Everett, E. F., and Rigler, L. G., *Radiol.*, 1952, v58, 524.
6. Christensen, W. R., and Sosman, M. C., *Am. J. Roentgenol.*, 1951, v66, 764.
7. Spencer, F. M., *Gastroenterology*, 1952, v21, 535.
8. Shehadi, W. H., *Am. J. Roentgenol.*, 1952, v68, 360.
9. McChesney, E. W., and Wyzan, H. S., unpublished data in the files of the Sterling Winthrop Research Institute.
10. Crismer, Roger, *Archives Internationales de Pharmacodynamie et de Therapie*, 1946, v72, 138.
11. Bang and Georg, *Acta Pharmacologica et Toxicologica*, 1948, v4, 87.

Received June 15, 1953. P.S.E.B.M., 1953, v83.

Suppression of Hyperoxic Convulsions by Nitrogen at High Pressure.*
(20412)

FRANK G. CARPENTER. (Introduced by W. O. Fenn)

From the Department of Physiology and Vital Economics, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Present information regarding the effect of breathing air at high pressures indicates that disturbances arise from the action of either oxygen or nitrogen on the nervous system(1). It is the purpose of this paper to present evidence that the depressant effect of nitrogen in this respect is actually antagonistic to the excitant action of oxygen when both gases are at increased pressure.

Method. Adult male and female mice were used in the experiments which were carried out in a steel chamber equipped with a window and large enough to accommodate 8 animals. Each mouse was isolated in an open ended glass jar standing upright on coarse wire mesh over soda lime. Approximately 2 minutes were required to fill the vessel to the desired gas pressure and the time for the appearance of the first convulsion in each mouse was recorded after the pressure of oxygen reached 90 psi.

Results. Usually the first indication of central nervous excitation in mice exposed to oxygen at high pressure is the presence of tail erection (Straub's sign) occurring at any time in the preconvulsive period. During the convulsive seizures clonic movements of the limbs are produced with such violence that the animal is propelled upwards against the restraining jar with considerable force. In males this is accompanied by ejaculation and in both sexes by urination. When the convulsion subsides the animal appears to be in no distress until the third or fourth seizure when obvious depression supervenes characterized by gasping-retching movements. The variation in convulsive behavior in mice after exposure to 90 psi of oxygen can be seen by reference to Fig. 1 (top) where the percent of animals involved in seizures is compared with the duration of their preconvulsive period. Convulsive behavior in mice first exposed to helium at 400 psi for 5 minutes as a control before the addition of 90 psi of oxygen is shown in Fig. 1 (middle). In both cases all animals displayed several seizures, as described above, before termination from respiratory failure. In mice first exposed to 400 psi of nitrogen for 5 minutes, before the subsequent addition of 90 psi of oxygen into the chamber, convulsions were produced in only 23 percent of the animals, and their median preconvulsive period was increased 44 percent beyond that of the control series in oxygen alone. Fig. 1 (bottom).

There is considerable difference between

---

*These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and The University of Rochester.

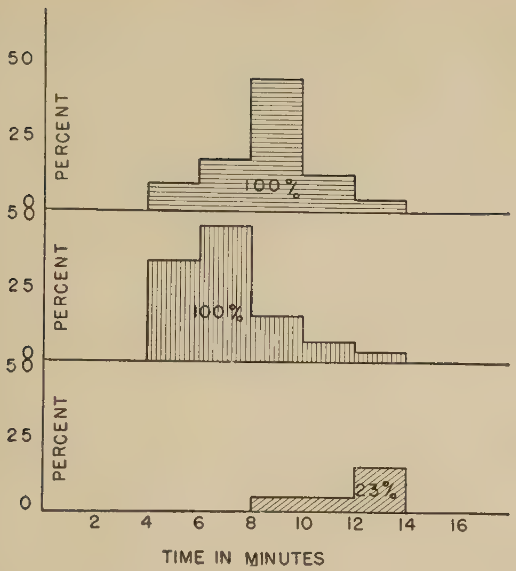


FIG. 1. Onset of convulsive activity with time in mice: Ordinate: % of total animals exposed. Abscissa: Time for appearance of first seizure. 25°C *Top:* At 90 psi of oxygen (36 animals). *Middle:* At 90 psi of added oxygen 5 min. after the mice were exposed to 400 psi of helium (33 animals). *Bottom:* At 90 psi of oxygen added 5 min. after the mice were exposed to 400 psi of nitrogen (42 animals).

convulsions produced in oxygen or helium-oxygen and those in nitrogen-oxygen. In the former case clonic discharges of the limbs become wild and rampant while in the latter instance the limb movements were restricted to twitches or tremors, but are indicated as convulsions in Fig. 1. The remaining 77% of the mice showed no recognizable convulsive activity whatsoever. In addition to the effects just described ejaculation and urination were never as copious as in the control animals. It should be emphasized however that the nitrogen treatment did not increase the survival or eliminate the pulmonary damage seen in the control group.

Discussion. One may note a shorter median preconvulsive period with helium-oxygen as compared with that for oxygen alone. The helium mixture due to its high thermal conductivity at this pressure (5.7 times that of water) may increase the susceptibility of the mice by lowering body temperature and initially increasing oxygen consumption. The latter condition is believed to potentiate oxygen toxicity according to the studies of Grossman and Penrod(2) and others(1). In measurements made in this laboratory an initial decline in rectal temperature amounting to .33°C/minute has been observed over a 20-minute period when mice are exposed to 400 psi of helium at 25°C. Oxygen consumption studies at this pressure and temperature however have not been made.

Bert(3) observed in dogs that tonic seizures caused by oxygen at high pressure were alleviated by chloroform and he therefore attributed them to exaggerated activity in the central nervous system. He noted a similarity between this action of oxygen and the symptoms of strychnine poisoning. Shilling and Adams(4) reported that rats given anesthetic doses of barbital or pentobarbital did not develop convulsive activity when exposed to OHP. Marks(5) has since demonstrated that several other CNS depressant drugs are capable of delaying the onset of hyperoxic seizures in mice and cats.

The depressant action of nitrogen at increased pressure reported by others(6,7) has recently been found to protect mice from electroshock seizures at pressures ranging from 220-400 psi(8). In these experiments it was observed that after a saturation period of 5 minutes the degree of protection afforded was proportional to the nitrogen pressure. It would seem therefore, that pressures beyond 400 psi of this gas would reduce still further the percentage of animals in our experiments showing hyperoxic CNS manifestations while practically no anticonvulsant action could be expected below 220 psi. However, it was of considerable interest that with the use of nitrogen alone, at this pressure, mice exhibit only slight depression in voluntary movements and are apparently able to right themselves or stand erect.

Summary. The time of onset and severity of convulsions was observed in 3 groups of mice exposed to 90 psi of oxygen, the first receiving the oxygen alone, the second 400 psi of helium plus oxygen and the third 400 psi of nitrogen plus oxygen. All the animals in groups one and two convulsed within 15 minutes of exposure to oxygen at this pressure but in the third group much less violent seizures appeared in only 23% of the mice and were delayed 44% in time of onset. Voluntary

movements and postural reflexes were not seriously disturbed by this pressure of nitrogen.

1. Bean, J. W., *Physiol. Rev.*, 1945, v25, 1.

2. Grossman, M. S., and Penrod, K. E., *Am. J. Physiol.*, 1949, v156, 177.

3. Bert, Paul, *La Pression Barometrique* 1878, translated by M. A. and F. A. Hitchcock, College Book Co., Columbus, Ohio, 1943.

4. Shilling, C. W., and Adams, B. H., *U. S. Navy Med. Bull.*, 1933, v31, 112.

5. Marks, H. P., Report prepared for the Subcommittee on Underwater Physiology of the Royal Naval Personnel Research Committee, Nat. Inst. for Med. Research, Hampstead, England, 1944.

6. Marshall, J. M., *Am. J. Physiol.*, 1951, v166, 699.

7. Lazarev, N. V., *Biological Actions of Gases Under Pressure*, Leningrad, Army-Navy Med. Acad., 1941.

8. Carpenter, F. G., *Am. J. Physiol.*, 1953, v172, 471,

Received June 15, 1953. P.S.E.B.M., 1953, v83.

Erythropoietic Stimulation Induced by "Anemic" Serum.* (20413)

A. ERSLEV, P. H. LAVIETES, AND G. VAN WAGENEN.

From the Departments of Internal Medicine and Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Conn.

Carnot and Deflandre(1) suggested that red cell production is governed by a factor present in blood serum. Since then numerous investigators have attempted to demonstrate this factor in serum and plasma from animals rendered anemic by bleeding or hypoxemic by exposure to air with a low oxygen tension(2). All the results were either negative or so equivocal that there has been a general refusal to accept the hypothesis of a humoral regulation of red cell production. Krumdiech(3) reported that 20 ml of serum from anemic rabbits induced a striking reticulocytosis when injected into normal rabbits. These results were not confirmed by Toha *et al.*(4) who subsequently injected 20 ml of "anemic" rabbit serum once a day for 3 days followed by 10 ml a day for 15 days into normal rabbits and were unable to demonstrate more than a slight rise in the number of reticulocytes. Erslev(5) was likewise unable to demonstrate a significant rise in reticulocytes when similar amounts of "anemic" plasma were used. However, when 200 ml of "anemic" rabbit plasma was administered to normal rabbits during a 4-day period a significant increase in the number of reticulocytes was found. The bone marrow showed a concomitant rise in the relative number of normoblasts and when 650 ml of "anemic" plasma was given during a period of 18 days there was a definite rise in hematocrit values and red blood cell count. These experiments showed that in rabbits red cell production can be stimulated by a humoral plasma factor. Reissman's(6) study with parabiotic rats tends to indicate that a similar factor exists in plasma from anemic rats.

Before further investigation was carried out into the nature and production of this factor it was felt advisable to demonstrate its presence in an animal species more closely related to humans than rabbit or rat. Macaca mulatta monkeys were selected for this purpose.

Materials and methods. Adult Macaca mulatta monkeys were used as donors and recipients. Three monkeys were made anemic by bleeding. They were bled from the femoral artery or vein after the animals had been anesthetized with 2.5 ml 6.0% nembutal given intravenously. Two or 3 times a week 80-100 ml of blood was withdrawn and "anemic" serum was obtained and pooled after the hemoglobin had been reduced to below 7 g/100 ml. Part of the pooled serum was kept for 2 weeks at 4°C while the remainder was frozen in a dry ice box. Serum from normal monkeys was ob-

---

*Supported by research grants from the U. S. Public Health Service and by a grant from the Fluid Research Fund of the Yale University School of Medicine.

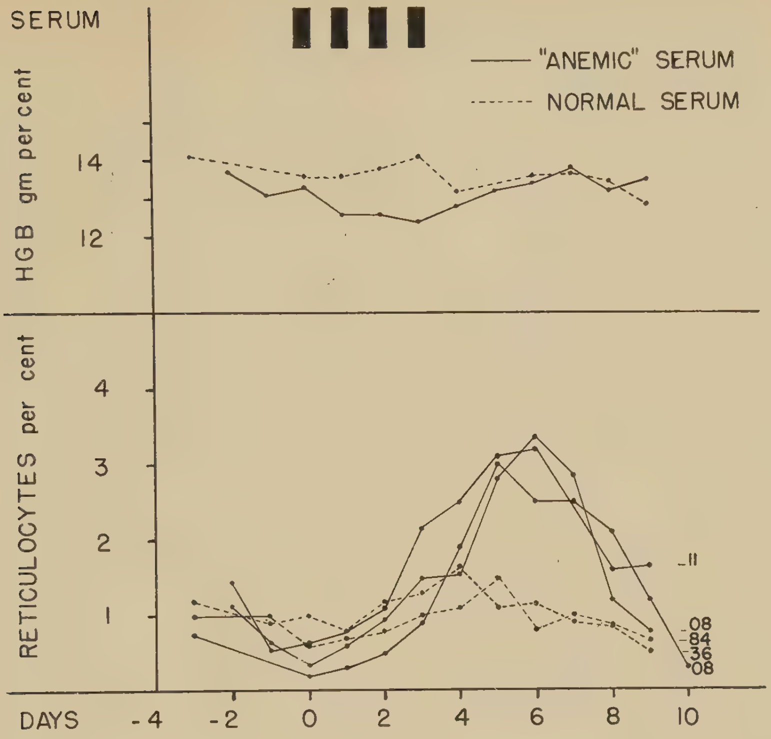


fig 1

FIG. 1. Upper part shows average hemoglobin of monkeys receiving "anemic" serum and monkeys receiving normal serum. Lower part shows the individual reticulocyte counts. Blocks at top of graph indicate the four days on which anemic or normal serum was injected intravenously.

tained in a similar manner and frozen. Four monkeys weighing between 3.5 kg and 5.3 kg were used as recipients. Serum was administered intravenously in the smaller saphenous vein once a day for 4 days. The total volume of serum given corresponded to from 6.1 to 10% of the body weight. This is the dosage which was found effective in rabbits(5). Determination of hemoglobin (electrophotometric) in duplicate, red blood cell count in duplicate, white blood cell count and platelet count were made daily on capillary blood from the ear. Reticulocyte counts (dry method) were determined by averaging the per cent obtained by two observers who counted 1000 red blood cells each.

Results. Monkey Mm-08, weighing 4 kg, received a total of 290 ml (7.3% of body weight) of "anemic" serum which had been kept at 4°C for 2 weeks. Three weeks later it received a total of 400 ml (10% of body weight) of "anemic" serum, which had been kept in a dry ice box for 5 weeks. Monkey Mm-11, weighing 5.2 kg, received 350 ml (6.7% of body wt) of "anemic" serum which had been kept at 4°C for 2 weeks. Fig. 1 shows that the "anemic" serum induced a significant rise in the number of reticulocytes in both animals.

Monkey Mm-84, weighing 4.5 kg, and Monkey Mm-36, weighing 4.7 kg, received 285 ml (6.4% and 6.1% of body weights) of normal serum which had been kept in a dry ice box for 2 weeks. Only a slight rise in the number of reticulocytes occurred (Fig. 1). The hemoglobin (Fig. 1), red blood cell count, white blood cell count and platelet count did not change significantly during or after the administration of normal or "anemic" serum.

Discussion. Serum obtained from anemic monkeys induced a definite rise in the number of reticulocytes when injected intravenously into normal monkeys but did not cause any other change in the peripheral blood count. Normal serum had no significant effect on the reticulocyte count or peripheral blood count. The reticulocyte count rose to approximately 3 times the normal values. This corresponds very closely to the relative increase observed in rabbits receiving "anemic" rabbit serum (5). Due to the crude semiquantitative technic and the small number of animals used it is impossible to evaluate the effect of freezing or refrigeration on the activity of "anemic" serum. However, the activity is not destroyed by these procedures. The above mentioned limitations also make impossible an evaluation of the effect of age of serum and of dosage on the reticulocyte response.

This experiment was designed to investigate the possible presence in the serum of anemic primates of a humoral factor capable of stimulating red cell production similar to the factor demonstrated in "anemic" rabbit plasma. The findings reported here strongly suggest that this is the case.

Summary. Serum from monkeys made anemic by bleeding induced a significant reticulocytosis when large amounts (from 6 to 10% of the body weight) were infused into normal monkeys. Serum from normal monkeys did not have a similar effect.

The authors wish to thank Miss Jean Greiner and Mr. Joseph Negri for valuable technical assistance.

---

1. Carnot, P., and Deflandre, C., *Compt. rend. Acad. d. sc.,* 1906, v143, 384.

2. Grant, W. C., and Root, W. S., *Physiol. Rev*, 1952, v32, 449.

3. Krumdieck, N., PROC. SOC. EXP. BIOL. AND MED., 1943, v54, 14.

4. Toha, J., Hodgson, G., and Weasson, E., *Boletin de la Soc. de Biol. de Concepcion,* 1952, v27.

5. Erslev, A., *Blood,* 1953, v8, 349.

6. Reismann, K. R., *Blood,* 1950, v5, 372.

Received June 15, 1953. P.S.E.B.M., 1953, v83.

Effect of Chemical Agents on Skeletal Content and Excretion of Injected Strontium^{89}.* (20414)

S. H. COHN, AND J. K. GONG. (Introduced by R. W. Brauer)

From the U. S. Naval Radiological Defense Laboratory, San Francisco, Calif.

It has been previously demonstrated(1) that administration of the sodium and calcium salts of ethylene diamine tetra-acetic acid (EDTA) results in the removal of substantial amounts of bone-fixed radioyttrium from rats. Removal effectively reduces the chronic radiation hazard associated with the internal deposition of this biologically long-lived fission product. Pretreatment of rats with the salts of EDTA also essentially prevented the deposition in bone of subsequently injected radioyttrium(1). The salts of EDTA are effective decontaminants because they are strong chelating agents which form a soluble undissociated complex with yttrium that is readily excreted through the kidney. Another chemical compound, zirconium citrate, has previously been found effective in reducing the skeletal concentration of yttrium and plutonium and in increasing their urinary excretion (2-4). Zirconium citrate also has a limited ability to increase strontium excretion from the body, if injected soon after the administration of the radioelement(5). The chemical agent, 2,3-dimercaptopropanol, (BAL) has been shown to increase the excretion of polonium and to mobilize it from bone into muscle tissue(6).

The object of the present experiment is to attempt the removal of injected radiostrontium from the bodies of rats by administration of the salts of EDTA, zirconium citrate, and

---

* The authors acknowledge the technical assistance of P. L. Moon and H. R. Gleason. The opinions or assertions contained herein are the private ones of the authors and not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

TABLE I. Experimental Outline.

Part of study	No. of rats	Chemical agent used in treatment	Time of treatment with reference to Sr^{89} administration	Sacrifice time post-Sr^{89} administration (day)
I	10	NaEDTA (10 mg) followed in 15 min. by CaEDTA (50 mg)	One hr before	3
	10	None (controls)	——	3
II	5	Zirconium citrate (25 mg Zr), see Schubert(2)	One hr before	5
	9	*idem*	" " after	5
	8	*idem*	15 min. before and twice daily for 3 days after	5
	10	None (controls)	——	5
III	7	BAL (0.04 ml of 10% sol. in peanut oil) followed in 30 min. by NaEDTA (10 mg) and CaEDTA (50 mg)	BAL given 15 min. before, followed by EDTA salts and both given twice daily for 3 days after	5
	14	Zirconium citrate (25 mg Zr), followed in 2 hr by NaEDTA (10 mg) and CaEDTA (50 mg)	Zirconium citrate given 1 hr before, followed by EDTA salts, then both given twice daily for 3 days	5
	14	None (controls)	——	5

BAL. BAL was used in combination with EDTA in this study in an attempt to remove bone-fixed radiostrontium. Strontium was selected as the radioactive material to be removed because of its high fission yield(7) and further, because it is a prototype of a large number of fission products, the alkaline earths.

Experimental. The experiment was performed in 3 parts, using Sprague-Dawley rats as test animals. All the rats were injected with 15 μc of carrier-free $Sr^{89}Cl_2$ and treated with various chemical agents as indicated in Table I. All injections were administered by the intraperitoneal route with the exception of BAL, which was given intramuscularly. Animals were kept in individual metabolism cages and fed *ad libitum* with Purina laboratory chow. The metabolism cages were suspended in large glass jars and the excreta collected in plastic bags fitted over the bottom of the cage. All animals were sacrificed at intervals of either 3 or 5 days as indicated in Table I. Both tibiae were removed, ashed, weighed, and dissolved in 1 N HCl. Aliquots were analyzed for their Sr^{89} content by use of a Geiger-Müller counter and standard scaling circuit, in a manner previously described(1). Excreta for each animal were ashed, dissolved in 1 N HCl, and analyzed similarly.

Results and discussion. Part I. No significant differences were found between the mean values of the body weight and tibia ash weight in the EDTA treated and control groups. It was observed that pretreatment with EDTA salts did not prevent injected Sr^{89} from depositing in bone, as these treated animals had the same level of bone Sr^{89} as did the controls. The mean strontium content of both the tibiae of the control and EDTA-treated animals 3 days post-Sr^{89} injection was equal to 1.59% of the injected dose. From these data, an estimate of the total skeletal Sr^{89} content was calculated by using a factor of 27 as the ratio of total skeletal Sr^{89} content to the Sr^{89} content of one tibia. This method was described in a previous study(1). The mean percentage injected dose in the skeleton was 42.9 ± 5.2% in the control group and 42.7 ± 3.5% in the pretreated group 3 days post-Sr^{89} injection. In previous experiments (1) it had been found that injection of EDTA prior to injection of the fission product Y^{91} is the most effective time for EDTA administration as far as ionic complexing ability is concerned. Thus, even in the most favorable situation, the salts of EDTA were without

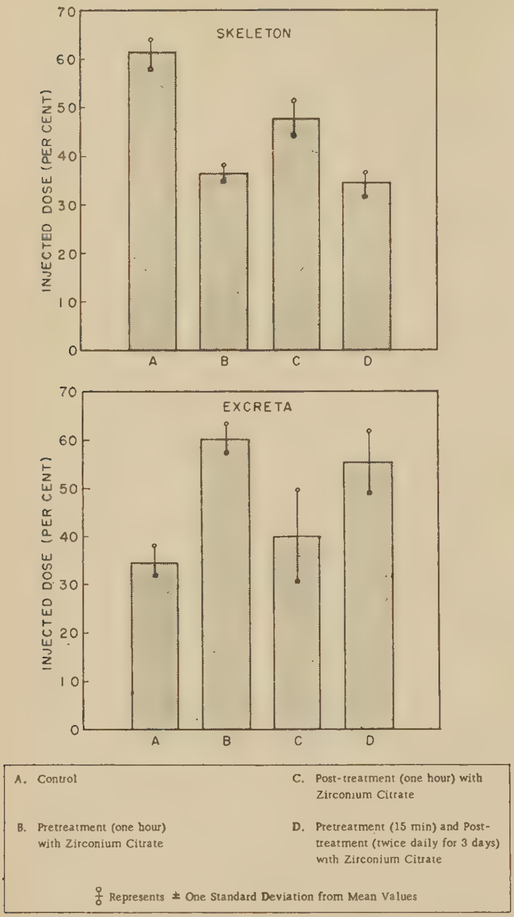


FIG. 1. Effect of zirconium citrate on skeletal content and excretion of injected radiostrontium.

effect on the skeletal content of radiostrontium. This could have been predicted largely on the basis that the stability constant of the strontium-EDTA complex at pH 7.4 is lower than that for the complex formed with the biologically competitive calcium ion(8).

Part II. The effects of zirconium citrate on the skeletal content and excretion of injected radiostrontium are presented in Fig. 1. In the animals pretreated with zirconium citrate and then injected one hour later with radiostrontium, the mean percentage injected dose of Sr^{89} in the skeleton after 5 days was 36.2% as compared with 61.1% for the untreated controls. The higher skeletal Sr^{89} content found in these controls as compared with that in Part I, is probably due to a difference in the age of the animals. It has been shown by Jones(9) that the uptake of injected Sr^{89} is an inverse function of age. The rats used in Parts I and III were mature animals 5 months old; the animals used in Part II were approximately 3 months old. Pretreatment in this experiment resulted in a skeletal Sr^{89} content 60% of that of the controls at 5 days post-Sr^{89} injection. Further, an enhanced Sr^{89} excretion of about 40% was observed when comparison was made with the Sr^{89} excretion of their controls. It is to be noted that the skeletal plus the excreta Sr^{89} content accounts for 95% of the total injected dose 5 days after Sr^{89} injection. It can, therefore, be concluded that most of the increased excretion was derived from that fraction of the injected Sr^{89} which would have ordinarily been fixed by the skeletal system.

In the animals treated with zirconium citrate one hour post-Sr^{89} injection, there was a less marked decrease in the skeletal Sr^{89} content (20%) and a smaller increase in Sr^{89} excretion than observed in the pretreated animals. In the animals pretreated with zirconium citrate and treated in addition twice daily for 3 days, the skeletal Sr^{89} was only slightly lower (56% of controls) than in the one-hour pretreated group. It would appear, therefore, that the effect of zirconium citrate in complexing and removing the Sr^{89} from the body is greatest when the zirconium citrate is administered before Sr^{89} injection. Continued injection of zirconium citrate for 3 days did not increase the excretion of Sr^{89} appreciably.

Strontium is chemically and metabolically very similar to calcium and is deposited in the bone salt, in contrast to yttrium which is deposited primarily in the uncalcified organic matrix of bone(10,11). Conditions which affect the metabolism of yttrium do not appear to influence the metabolism of strontium(9). Thus, as Schubert(2) points out, one would not necessarily expect zirconium administration (found to influence the metabolism of yttrium) to greatly affect the metabolism of bone-fixed strontium. Schubert did find, however, that after strontium uptake by bone is complete, the administration of zirconium citrate can apparently remove a small portion of the strontium from the body(5). The principal action of the zirconate complex is

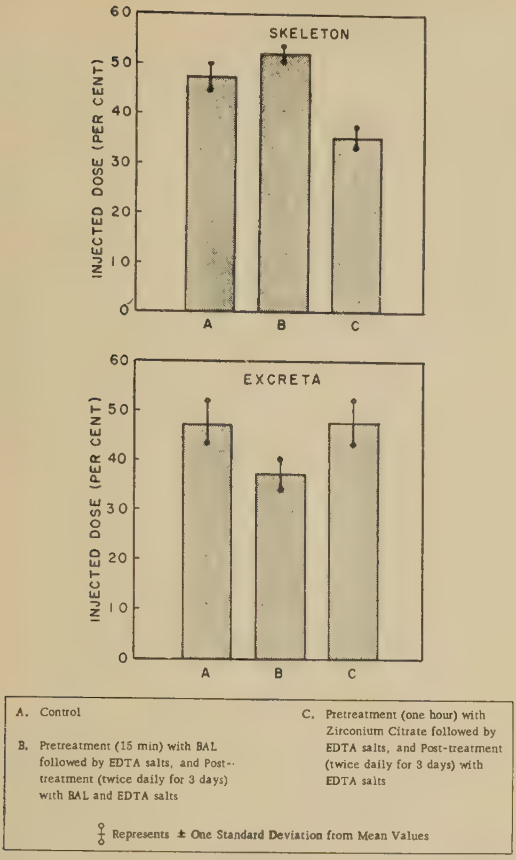


FIG. 2. Effect of various chemical agents on skeletal content and excretion of injected radiostrontium.

then largely on that fraction of injected Sr^{89} deposited in the soft tissue. The fact that pretreatment with zirconium did have an effect on the disposition of subsequently injected strontium may possibly be explained in the following manner. Uptake of small amounts of strontium by bone is usually interpreted as a result of adsorption or ionic exchange(12). If injected zirconium citrate is adsorbed on the surface of bone salt crystals, so that free surface-bonds are saturated, the subsequently injected strontium would not be fixed by the bone and would then be excreted. This same explanation would hold for the animals treated with zirconium citrate at one hour after strontium injection. At one hour post-intraperitoneal injection, strontium is still largely in the blood and soft tissue.

Part III. The results of EDTA treatment used in combination with zirconium citrate and with BAL are presented in Fig. 2. Treatment with BAL prior to sodium and calcium EDTA administration had no significant effect on the skeletal Sr^{89} or on the amount found in the excreta 5 days post-Sr^{89} injection. The total percentage recovery of the injected dose in the experimental animals was somewhat lower than that found in the controls. The rationale for pretreatment with BAL was the observation that BAL is able to shift polonium from the skeleton to the soft tissue(6). It is apparent that BAL does not have this ability to shift strontium from the bone to the soft tissues where it would be available for complexing with administered EDTA salts.

If the removal of strontium from the skeleton following the combined administration of EDTA and zirconium citrate is compared with the results of the experiment in which zirconium citrate alone was administered, it is seen that the combined use not only fails to enhance the effect of zirconium citrate, but on the contrary lowers it. This latter effect may be due to the complexing of zirconium by EDTA with subsequent removal from the body.

It can be concluded, then, that the salts of EDTA administered before or after Sr^{89} injection, either alone or in combination with zirconium citrate or BAL, have no effect on the Sr^{89} content in the skeleton or on the concomitant Sr^{89} excretion.

Summary. 1. The effect of chemical agents ethylene diamine tetra-acetic acid (EDTA) and zirconium citrate on skeletal content and excretion of injected radiostrontium was studied in rats. BAL was also administered in combination with EDTA in an effort to increase the excretion of injected strontium. 2. Administration of salts of EDTA under the most favorable conditions for its action had no effect on skeletal distribution of injected carrier-free Sr^{89}. Zirconium citrate administrated prior to injection of Sr^{89} resulted in a level of Sr^{89} in the skeleton at 5 days 40% lower than that found in controls. A corresponding increase in Sr^{89} excretion was also noted. Combined administration of EDTA with zirconium citrate and with BAL are without effect on skeletal distribution of injected Sr^{89}. Possible

explanations for the effect of the various chemical agents employed are discussed.

1. Cohn, S. H., Gong, J. K., and Fishler, M. C., *Nucleonics,* 1953, v11, 56.

2. Schubert, J., *J. Lab. and Clin. Med.,* 1949, v34, 313.

3. Schubert, J., and White, M. R., *J. Biol. Chem.,* 1950, v184, 191.

4. White, M. R., and Schubert, J., *J. Pharm. and Exp. Therap.,* 1952, v104, 317.

5. Schubert, J., and Wallace, H., *J. Biol. Chem.,* 1950, v183, 157.

6. Hursh, J. B., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 210.

7. Siegel, J. M., *J. Am. Chem. Soc.,* 1946, v68, 2411.

8. Schwarzenback, G., and Ackermann, H., *Helv. Chem. Acta,* 1948, v31, 1029.

9. Jones, D. C., and Copp, D. H., *J. Biol. Chem.,* 1951, v189, 509.

10. Copp, D. H., Axelrod, D. J., and Hamilton, J. G., *Am. J. Roentgenol.,* 1947, v58, 11.

11. Hamilton, J. G., *Rad.,* 1947, v49, 325.

12. MacDonald, N. S., *et al.,* Univ. of Calif. at Los Angeles, Report UCLA-222.

Received June 16, 1953. P.S.E.B.M., 1953, v83.

Safety of Immune Serum Globulin with Respect to Homologous Serum Hepatitis.* (20415)

RODERICK MURRAY AND FRANK RATNER (Introduced by S. E. Branham)

From the Laboratory of Biologics Control, National Microbiological Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Md.

Immune serum globulin is prepared commercially at the present time by two general processes: (a) salting-out, *e.g.,* by means of ammonium sulphate, and (b) by the cold-ethanol process(1,2) or a variation thereof. Blood obtained either by venipuncture or by extraction from placentas is used as the source material. *The Minimum Requirements of the National Institutes of Health for Immune Serum Globulin*(3) have stipulated that pools consist of at least 500 individual contributions.† In view of the fact that approximately one individual out of 300 receiving blood transfusions may develop hepatitis(4-7), it might be expected that many of the pools of plasma used in the manufacture of immune serum globulin would be infected with the agent(s) of homologous serum hepatitis. General experience indicates that the incidence of homologous serum hepatitis following administration of immune serum globulin must be low. According to Ordman *et al.*(8), one of 400 individuals in one series receiving immune serum globulin developed jaundice. The fact that 74 other children in the series received material from the same lot without untoward sequelae makes it unlikely that the immune serum globulin was the cause of the hepatitis in this one individual. During 1943-44 a follow-up by Janeway of 869 individuals who received immune serum globulin revealed no cases of jaundice(9). Hammon, Coriell, and Stokes, in a follow-up of field studies on poliomyelitis in Utah, found that immune serum globulin injected into 2,800 children was not icterogenic(10). Material prepared by the cold-ethanol method was used in these studies. Cockburn(11) has reported the development of one case of hepatitis among 58 subjects inoculated with immune serum globulin produced by ether fractionation(12). The plasma used in its preparation gave rise to 7 cases of hepatitis among 10 children who were given from 5 to 30 ml parenterally.

---

* This investigation was conducted in part under contract from the Office of the Surgeon General, Department of the Army, and under the sponsorship of the Commission on Liver Disease, Armed Forces Epidemiological Board. This work was made possible through the cooperation of the Bureau of Prisons, U. S. Department of Justice and of the Staff of the U. S. Penitentiary, Lewisburg, Pa. The service rendered by the volunteers is gratefully acknowledged.

† A recent revision (April 9, 1953) of these Minimum Requirements has increased the figure to 1,000. This is also the figure laid down by the Minimum Requirements for Poliomyelitis Immune Globulin (Apr. 9, 1953).

Because of the large amounts of immune serum globulin which are being used and because of the small number of reported follow-up studies, it was deemed advisable to determine the infectivity of immune serum globulin with reference to the agent(s) of homologous serum hepatitis by inoculation studies in human volunteers. This was part of an extensive program concerned with the study of the safety of blood and blood products, the general conduct of which is being reported elsewhere(13).

Methods. Material from a single large pool of infected plasma, approximately 130 liters in volume, was used in all of the studies on the safety of plasma and its derivatives. Approximately 16 liters of this plasma were fractionated‡ by the cold-ethanol process, utilizing a combination of Methods 6 and 9 of Cohn *et al.*(1,2). The immune serum globulin so prepared was subjected to the required tests for sterility, safety, and pyrogenicity(3). This material was then inoculated subcutaneously into 10 volunteer subjects, each of whom received 2.0 ml. Simultaneously 5 volunteers in a control group each received 1.0 ml of the original plasma from which the globulin had been produced.

Results. None of the subjects who received globulin developed hepatitis, with or without jaundice, while one of the 5 in the control group developed hepatitis with jaundice having an incubation period of 84 days. A second individual in this control group developed positive hepatic tests suggestive of hepatitis without jaundice.

Additional evidence of the infectivity of the original plasma was provided by the results of a concurrent study designed to determine the infective titer of the plasma. In the course of this, 5 subjects were inoculated with 1.0 ml amounts of a 1/1000 normal saline dilution of plasma from the same vial as that administered to the control group above. All inoculations were performed at the same time. Two cases of hepatitis with jaundice developed in this third group of volunteers. Incubation periods were 89 and 127 days.

Summary. Immune serum globulin produced by the cold-ethanol method from proved infectious plasma failed to produce hepatitis in 10 volunteer subjects inoculated with 2.0 ml each.

1. Cohn, E. J., Strong, W. L., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

2. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr., *J. Am. Chem. Soc.*, 1949, v71, 541.

3. Minimum Requirements: Immune Serum Globulin (Human) 2nd Revision, Nov. 20, 1946. National Institutes of Health.

4. Spurling, N., Shone, J., and Vaughan, J., *Brit. M. J.*, 1946, v2, 409.

5. Lehane, D., Kwantes, C. M. S., Upward, M. G, and Thomson, D. R., *Brit. M. J.*, 1949, v2, 572.

6. McGraw, J. J., Jr., Strumia, M. M., and Burns, E., *Am. J. Clin. Path.*, 1949, v19, 1004.

7. Allen, J. G., Sykes, C., Enerson, D. M., Moulder, P. V., *J. Lab. and Clin. Med.*, 1950, v36, 796.

8. Ordman, C. W., Jennings, C. J., Jr., and Janeway, C. A., *J. Clin. Invest.*, 1944, v23, 541.

9. Janeway, Charles A., *Bull. N. Y. Acad. Med.*, 1945, v21, 202.

10. Hammon, William McD., Coriell, Lewis L., and Stokes, Joseph, Jr., *J. Am. M. Assn.*, 1952, v150, 739.

11. Cockburn, W. C., Harrington, J. A., Zeitlin, R. A., Morris, D., and Camps, F. E., *Brit. M. J.*, 1951, v2, 6.

12. Kekwick, R. A., and Mackay, M., *1st. Int. Congr. Biochemistry*, Cambridge, Abstract of Communications, 1949, 147.

13. Murray, R., *et al.*, to be published.

Received June 17, 1953. P.S.E.B.M., 1953, v83.

---

‡ Fractionation was carried out by E. R. Squibb & Son.

Effects of Caesarean Delivery upon Maternal Behavior in Rats.* (20416)

JERRY LABRIOLA. (Introduced by F. A. Beach)
From the Department of Psychology, Yale University.

Experience in building nests and caring for young is not essential for biologically effective maternal behavior in laboratory rats, for primiparous females reared in isolation bear and rear young successfully (Sturman-Hulbe and Stone(1); Beach(2). Wiesner and Sheard (3) reported that female rats will display maternal behavior even though their young are removed by caesarean section, but this experiment lacked several necessary controls and there were no quantitative measures of the behavior. The present study was conducted to compare the maternal reactions of primiparous females that gave birth normally with those of females delivered by caesarean operation.

Methods. Twenty virgin female rats of the Sprague-Dawley strain were mated to males and placed in individual observation cages 2½ days before parturition was expected. The cages were 3 feet square and 2 feet deep, and 100 strips of paper toweling were suspended from the walls in such a way that the rat could easily detach them for use in nest building. Twenty-four hours after the animals were put in the cages, records were made of the number of paper strips detached and the grade of nests built. At this time 10 rats constituting the experimental group were subjected to caesarean operation and then returned with their young to the observation cages. Since the experimental rats did not lactate normally their young received little or no nourishment. Therefore, to make sure that these females would be stimulated by active babies during the retrieving test, each experimental animal was given a new, foster litter every 24 hours. The pregnant control rats remained in the cages and gave birth normally. Twenty-four hours after the young had been delivered, either surgically or naturally, the first retrieving test was conducted. The mother was removed from the cage, the young were scattered about the cage floor and the female's behavior after being returned to the cage was recorded. If a female failed to retrieve within 15 minutes, the young were replaced in the nest and another test was conducted 24 hours later. This procedure was repeated until retrieving occurred or until 72 hours had elapsed since parturition. Twenty-four hours after the final retrieving test the nest was destroyed, new paper strips were hung from the walls, and the number of strips detached and grade of nest built were recorded the following day.

Ten nulliparous (*i.e.* non-pregnant and non-lactating) females constituted a second control group. These rats were supplied with foster young and given the same tests as the other groups.

Results. Before parturition all of the pregnant control animals and 7 of the 10 experimental rats detached an average of 90 of the 100 paper strips. The remaining 3 experimental cases and all 10 of the nulliparous females detached an average of only 24 strips.

In the post-parturitional nest-building test the differences persisted, with 10 controls and the same 7 experimental animals detaching an average of 98 strips as contrasted with a mean of 26 strips for the 10 nulliparous and 3 of the experimental rats. The grade of nest built corresponded to the amount of material used. The nests of all nulliparous and 3 of the experimental rats were scored "poor," whereas the 7 active experimental females and 9 of the 10 lactating controls were rated as "excellent." One control nest was scored "good."

None of the nulliparous females retrieved foster young in any of the 30 tests in which they were observed, and retrieving was absent in the 3 experimental rats which had failed to build nests. All of the 10 control and 5 of the experimental females retrieved readily in the first test, 24 hours post-partum. One experimental case did not respond in the first test but did so 48 hours post-partum, and one

---

* This research was supported in part by a grant to Dr. Frank A. Beach from the Committee for Research on Problems of Sex, N.R.C.

more experimental rat retrieved only after 72 hours. There was no observable difference in the nature of the retrieving behavior of the normally-delivered mothers and the caesarean cases.

These results suggest that the process of giving birth and associated activities such as cleaning the young and eating the placenta are not essential precursors to post-parturitional nest building and caring for the young. The fact that 3 of the experimental females failed to display maternal behavior may have been due to one or all of several factors. They may have suffered more severe shock as a result of the operation than did the remaining 7 rats in this group. They may have been resistant to the stimulus of foster young in the retrieving tests. Or they may have been less "maternal" even before the operation. It has been reported that some otherwise normal female rats will not build nests or care for their young (Beach,1937). The 3 negative experimental animals in the present experiment may belong in such a category. Their failure to build nests prior to operation is evidence in favor of such an interpretation.

Regardless of the explanation for the 3 non-maternal animals, the occurrence of normal nest-building and retrieving in 7 experimental cases supports the conclusion advanced above. Furthermore, since the experimental females probably did not lactate normally it can be added provisionally that secretion of milk and nursing are not necessary for maternal responsiveness. Since mere contact with new-born rats did not evoke maternal reactions in the nulliparous controls it appears probable that the key factors involved in maternality are the hormonal changes associated with pregnancy and its termination.

---

1. Sturman-Hulbe, M., and Stone, C. P., *J. Comp. Psychol.*, 1929, v9, 203.

2. Beach, F. A., *J. Comp. Psychol.*, 1937, v24, 393.

3. Wiesner, B. P., and Sheard, N. M., 1933, Maternal Behavior in the Rat, Edinburgh, Oliver and Boyd.

Received June 12, 1953. P.S.E.B.M., 1953, v83.

Pathologic Physiology of Mammalian Blood Platelet Utilizing P^{32} Tagged Rabbit Platelets.* (20417)

JOHN F. MUELLER.

From the Department of Medicine, University of Cincinnati and Cincinnati General Hospital.

Recently much attention has been focused on the etiology and pathologic-physiology of thrombocytopenia. One of the aspects studied has been the life span of the normal platelet in patients with various types of thrombocytopenia. This was accomplished by transfusing platelet-rich polycythemic blood into persons with thrombocytopenia. The work was begun in an effort to perfect a method for the study of platelet survival in the rabbit prior to attempting a similar study in human beings. During the course of the investigation some interesting observations concerning the physiology of the platelet have been made. In addition, a new technic is described which may be of value in future studies of this cell.

Weisberger and Heinle(1) have utilized the technic of isotopic labelling to study the survival rate of transfused leukocytes. A similar procedure has been devised for the platelet utilizing radioactive phosphorous. Julliard (2) and his co-workers in France have very recently published some data on the fate of transfused radioactive platelets. Their results are comparable to those herein reported.

Methods and results. Unagglutinated platelet suspension, essentially free from erythrocytes and leukocytes are prepared in the following manner. Silicon technic is used throughout the procedure. 45 cc of rabbit heart blood collected in 5 cc of Na Sequestrene is centrifuged at 800 rpm for 15 minutes. The platelet rich plasma is then transferred to

---

* This study was aided in part by a grant from the National Institutes of Health, Public Health Service.

especially manufactured platelet isolation tubes.† These are wide-mouthed, narrow-stemmed tubes which allow the buffy coat to be extracted readily with capillary pipettes. The tubes are spun, in the cold, at 3000 rpm for 20 minutes. The platelet layer is removed, resuspended in cold saline and centrifuged again. Usually 3-4 such centrifugations suffice. The final product is suspended in 5 cc of cold saline. With this method a 40-60% yield is considered satisfactory, since a considerable loss is necessary to assure purification. Fifteen-twenty microcuries of inorganic radioactive phosphorus is then added to the platelet suspension. This preparation is gently agitated at 37°C overnight. The following morning the platelets are washed 3 times with cold saline, again using high speed centrifugation in the cold. The platelets which are now labeled with radioactive phosphorus are finally suspended in 5 cc of cold saline and are ready for whatever testing is to be done. The platelet count of the final preparation averages 500000 to 1000000 platelets per cmm. Aliquot specimens of each wash water as well as the final specimen are taken for radioactive counting. All radioactive counting is done using a Geiger-Mueller counter with a mica end window and is recorded as beta counts per minute per given amount of specimen. As stated above aliquots were always saved so that no correction was necessary for decay. Originally the platelets were tagged *in vivo* by injecting inorganic radioactive phosphorus intravenously in rabbits and then removing the platelets after 48-72 hours. However the magnitude of the activity acquired by the platelets with such a procedure was so low that *in vitro* methods were necessary. This can be appreciated by referring to Table I. The difference between the two methods is obvious, as well as the fact that the platelets are capable of incorporating considerable radioactivity.

TABLE I. Example of Typical Experiment Showing that Platelets Readily Incorporate Radioactivity after *In vitro* Incubation with P^{32} as Compared to *In vivo* Tagging.

	Counts/min./1 ml	
Specimen	*In vitro* tagging	*In vivo* tagging
1st wash	21644	795
2nd "	8744	32
3rd "	3464	16
Platelet suspension	105284	72

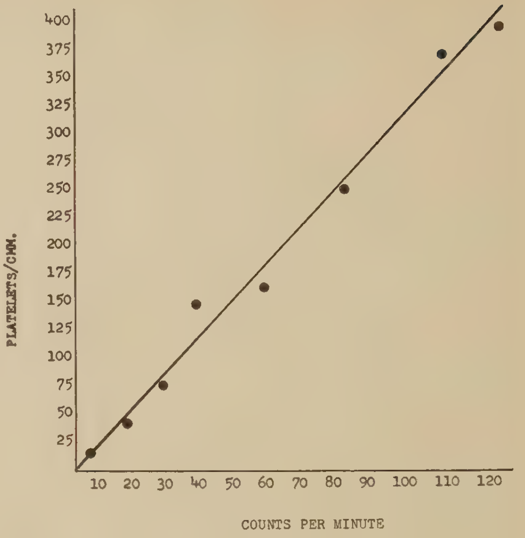


FIG. 1. Correlation between absolute platelet counts and radioactivity.

Results. In an attempt to demonstrate specificity of activity in the platelets tagged *in vitro* the radioactive count of such a preparation was correlated with decreasing numbers of platelets. A direct relationship is present as is shown in Fig. 1. Furthermore the radioactivity of the wash water levels off after 3 washings and ordinarily contains less than 5% of the activity present in the platelets.

For an isotopic labelling technic to be valid one must not only prove specificity of activity but also demonstrate incorporation of the P^{32} into the cell. If overnight *in vitro* incubation dislodges the radioactivity from the platelets the use of such a technic for studying survival rates is hampered seriously. This was found to be the case initially when the platelets were incubated with P^{32} for only 3 hours. However this was not the case when the length of incubation was increased to 14 hours. In this instance the radioactivity remained associated with the platelet (Table II).

This information seemed to indicate that the platelets were actively incorporating the phosphorus. Since very little is known about

---

† Obtained from E. Machlett, 220 E. 23rd St., New York City.

TABLE II. Demonstration that Prolonged *In vitro* Incubation Firmly Incorporates P^{32} into Platelet as Compared to Shorter Incubation Periods.

Specimen	Counts/min./1 ml After 3 hr incubation	 After 14 hr incubation
1st wash	10260	7440
2nd "	1080	2136
3rd "	720	1950
4th "	636	
5th "	540	
Platelet suspension	11700	21360
Wash water after overnight incubation	10680	2430
Overnight platelet suspension	1020	18930

TABLE III. Demonstration Depicting Kinetics of Assimilation of P^{32} into Rabbit Platelet after Varying Periods of *In vitro* Incubation.

Specimen		Counts/min./1 ml After 3 hr incubation	 After 14 hr incubation
Phosphorous compartment	Acid sol. P.	4140	2580
	Fat sol. P.	120	2460
	Organic P.	72	20460

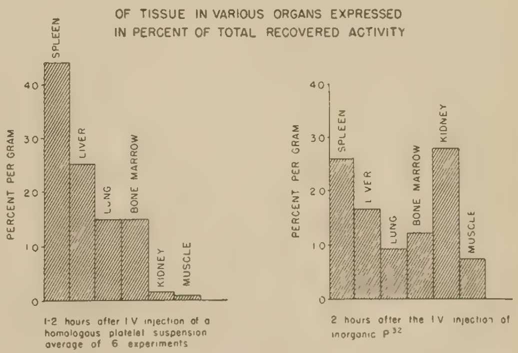
TABLE IV. Average Recovery of Radioactivity in Blood of 10 Rabbits Following Intravenous Injection of Homologous Radioactive Platelet Suspension.

Sample of blood	% of inj. activity/ ml blood	% of inj. activity totally recovered/ml (blood vol, 250 cc approx.)
Immediate (theoretical)	.4	100
1-3 min.	.14	34
2 hr	.01	3

platelet metabolism it was decided to study in more detail the kinetics of assimilation of the P^{32} into the cell. The 3 phosphorous compartments, acid-soluble, lipid and organic, were explored for radioactivity by subjecting the tagged platelets to simple extraction procedures. Eight cc of cold 10% trichloracetic acid added to 4 cc of the platelet suspension removed the acid soluble phosphorus. The precipitate was then treated with a 2:1 hot alcohol-ether mixture which extracted the lipophorphorous components. That which remained was dissolved in 2 N KOH. Aliquots of these extracts were subjected to Geiger counting. The results are given in Table III. After 3 hours incubation with P^{32} most of the radioactivity is in the acid-soluble portion which accounts for the ease with which the P^{32} moved in and out of the platelets. However after 14 hours (overnight) most of the phosphorus is in the organic compartment where it is more tightly bound. From this set of experiments it would appear that the labelling technic herein described is applicable to a study of the life span of transfused rabbit platelets.

Therefore radioactive rabbit platelet suspensions containing 500000 to 1000000 platelets per cmm and representing between 200000 and 700000 beta counts/minute were reinjected into an ear vein of the same animal from which they had previously been isolated. Platelet counts performed on the rabbit's blood before and after the transfusion failed to reveal any consistent change. The radioactivity of the peripheral blood was followed by obtaining 1 cc specimens of heart blood for Geiger counting at varying intervals after the injection. In most instances the interval was 2 hours although some samples were taken at 4 and 24 hours. In the latter cases there was no variation from that obtained at two hours. It is apparent from Table IV that the activity disappeared from the blood rather promptly and in fact was almost completely gone after 2 hours.

At the end of 2 hours the animals were sacrificed, various organs removed *in toto* when possible, and aliquot portions ashed and counted for radioactivity. The recovered activity was found distributed primarily in the organs of high reticulo-endothelial activity,



1-2 hours after I V injection of a homologous platelet suspension average of 6 experiments

2 hours after the I V injection of inorganic P^{32}

FIG. 2.

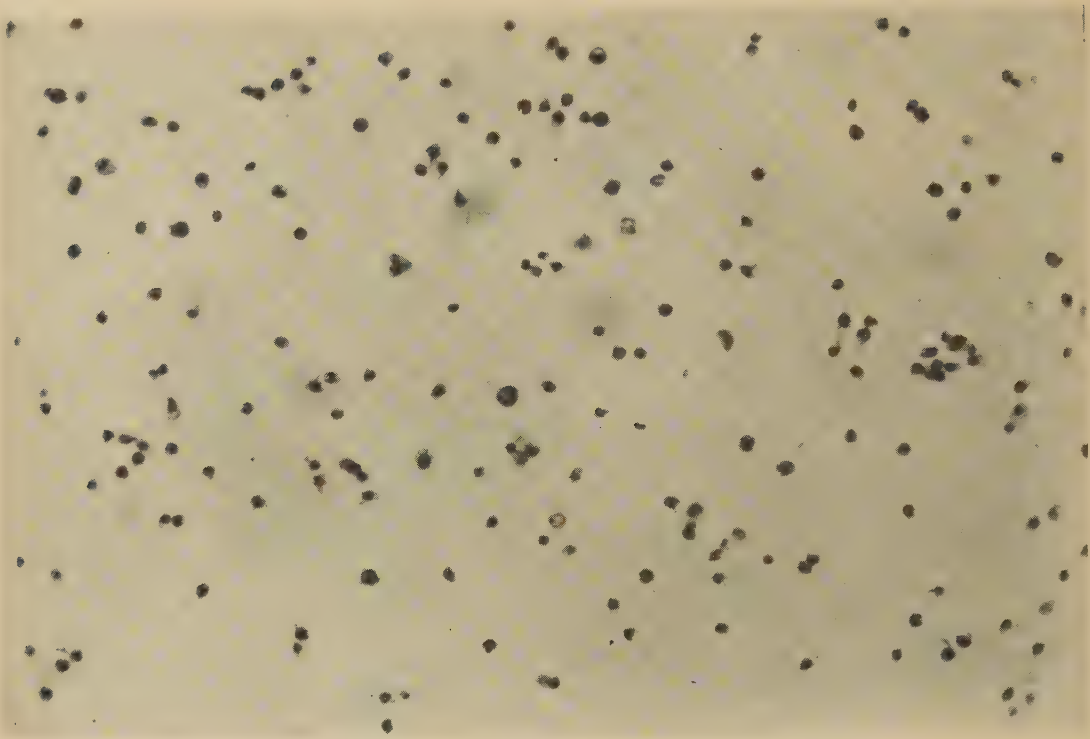


FIG. 3.

the spleen, liver, lung and bone marrow in that order (Fig. 2) when expressed in terms of recovered activity per gram of tissue.

These data would indicate that under the conditions of the experiment transfused radioactive platelets disappear from the circulating blood quite rapidly in the normal rabbit and that the reticulo-endothelial system, in particular the spleen, plays a prominent role in this process.

The following tests were performed on the radioactive platelets to determine viability. 1) The platelets stained with brilliant cresyl blue have normal morphology (Fig. 3). 2) Clots prepared from purified fibrinogen retract upon the addition of radioactive platelets. However these platelets will not restore retraction to a natural clot prepared from platelet-free plasma. 3) The abnormal serum prothrombin time of platelet-free plasma is restored to normal upon the addition of radioactive platelets (Table V). 4) Endogenous respiration as measured in a Warburg does occur, but is greater in the treated than in the non-treated platelets. This observation may indicate that some damage has occurred during manipulation.

Discussion. In recent experiments performed by several investigators, the life span of the human platelets has been estimated to be 3-6 days(3-5). This figure has been derived by the indirect method of following the platelet count of human subjects following the transfusion of platelet rich blood or platelet suspensions into these individuals. Earlier work in animals by Duke(6) had indicated that the life span of platelets is measured in days rather than minutes or hours. The present report does not agree with these findings. The immediate question arises: are these platelets, which admittedly go through a traumatic procedure "normal"? The results of the viability tests reported above indicate

TABLE V. Serum Prothrombin Time.

Material	Time, sec.
Native serum	150
Serum from platelet-free plasma	16
Serum from platelet-free plasma plus radioactive platelets	180

that the radioactive platelets have been damaged to some degree.

It is obvious that more investigation must be done before a final answer can be given but these experiments do indicate the sites where platelets may be destroyed and point up certain aspects of platelet metabolism heretofore not demonstrated. It is hoped in the future that this method may be applied to studies in human beings.

Summary. 1. Purified, unagglutinated platelet suspensions prepared from rabbit blood do incorporate radioactive phosphorus readily, and therefore lend themselves to life span studies *in vivo*. 2. These studies indicate that transfused rabbit radioactive platelets are rapidly removed from the peripheral blood and the radioactivity recovered in organs of the reticulo-endothelial system, namely spleen, liver, lung and bone marrow. 3. The problem of viability of these platelets is discussed. The evidence suggests that damage has occurred during manipulation. 4. These observations are important primarily in demonstrating aspects of metabolism and destruction of platelets.

The author wishes to thank Dr. Eugene Saenger of the Radioisotope Laboratory of the Cincinnati General Hospital for his valuable advice and assistance in obtaining and using the radioactive isotope.

---

1. Weisberger, A. S., Heinle, R. W., Storraasli, J. P., and Hannah, R. J., *J. Clin. Invest.*, 1950, 336.
2. Julliard, J., Maupin, B., Chary, R., Theilleux, R., Nau, P., and Loverdo, A., *Societe de Biologie,* 1952, 211-214.
3. Hirsch, E. O., Favre-Gilly, J., and Dameshek, W., *Blood,* 1950, v5, 568.
4. Hirsch, E. O., and Gardner, F. H., *J. Clin. Invest.*, 1951, v30, 649.
5. Stefanini, M., Chatterjea, J. B., Dameshek, W., Zannos, L., Santiago, E. P., *Blood,* 1952, v7, 53.
6. Duke, W. W., *J. Exp. Med.*, 1911, v14, 265.

Received April 9, 1953. P.S.E.B.M., 1953, v83.

Reversible Inhibition of Cell Division and Enlargement in Plant Tissues by 2, 6-Diaminopurine.* (20418)

Carlos Miller. (Introduced by Folke Skoog)

From the Department of Botany, University of Wisconsin, Madison, Wis.

Since the report by Hitchings and coworkers(1) that 2,6-diaminopurine (DAP) inhibits the growth of *Lactobacillus casei*, the compound has been used in many studies of growth in microorganisms and animals as well as in virus reproduction. In survey experiments, DeRopp(2) tested the chemical for action on growth of plant tumor tissue but observed no effects. In this paper are reported the results of experiments with several different tissues of higher plants in which DAP inhibits cell division—as it does in animals and microorganisms—and also inhibits cell enlargement and the formation of vegetative buds.

Cell division in tobacco stem segments. Sterile tobacco stem segments were obtained from 3 to 4 ft tall tobacco plants, Wisconsin No. 38, grown in a greenhouse. After removal of the leaves, the stems were cleansed by swabbing with 95% ethyl alcohol and then the outer layers down to the cambium were peeled off. The remaining cylinders were cut into sections 1 cm long. Three to 4 segments were obtained from each section by cutting it tangentially and the central core (pith) was discarded. These stem segments contained parts from the vascular-cambial region, xylem, internal phloem, and pith and averaged about 6 mm in width, 10 mm in length, and 2 mm (maximal) in thickness. They were placed pith side down on the surface of the basal or

---

* Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council and in part by the Research Committee of the Graduate School on funds from the Wisconsin Alumni Research Foundation.

TABLE I. Inhibition of Budding and Callus Formation in Tobacco Stem Segments by 2,6-diaminopurine (DAP); Reversal by Adenine Sulfate.*

Addenda					
DAP (mg/l)	Adenine sulfate (mg/l)	No. of segments	Fresh wt of callus/segment (mg)	% of segments with buds	No. of buds/segment
—	—	30	79	97	3.9 ± .3†
—	80	27	82	100	6.6 ± .6
1	—	27	10	0	0 ± .0
1	80	30	85	100	5.1 ± .3
5	—	30	4	0	0 ± .0
5	80	30	22	47	.8 ± .2

* Readings made 39 days after start of test.
† Stand. error.

TABLE II. Inhibition of Budding and Callus Formation in Tobacco Stem Segments by 2,6-diaminopurine (DAP) (0.5 mg/l); Attempted Reversal with Various Compounds (3×10^{-4}M).*

Addenda					
DAP	Compound	No. of segments	Fresh wt of callus/segment (mg)	% of segments with buds	No. of buds/segment
—	——	21	108	100	4.5 ± .3†
—	Adenine	24	114	100	6.8 ± .7
—	Guanine	18	118	100	5.7 ± .5
+	——	24	57	46	1.4 ± .4
+	Adenine	24	120	100	6.3 ± .5
+	Arginine	21	65	57	1.4 ± .3
+	Guanine	24	76	67	2.1 ± .4
+	Hypoxanthine	21	82	86	2.5 ± .3
+	Thymine	24	55	58	1.2 ± .3
+	Xanthine	18	68	72	1.5 ± .3

* Readings made 37 days after start of test.
† Stand. error.

test media. The basal medium was modified White's solution as previously described(3) except that the level of KH_2PO_4 was increased from 12.5 to 37.5 mg/l and was made up with 1% Bacto-agar. The pH of each solution was adjusted to about 4.3 before autoclaving. Three stem segments were cultured aseptically on 50 ml of the medium in a 125-ml Erlenmeyer flask. Ten flasks or a total of 30 segments were prepared for each treatment.

On the control medium, these stem segments develop a white callus located mainly at the basal ends. This callus is easily cut from the old stem parts. In the experiment represented in Table I, the mean fresh weight of this callus after 39 days was 79 mg per segment. In the presence of 1 mg/l 2,6-diaminopurine, the fresh weight of the callus was reduced to 10 mg per segment. The fresh weight was only 4 mg in the presence of 5 mg/l DAP. A reduction in the thickness of the callus and the area covered by the callus occurred at both concentrations. In other experiments, callus formation was completely prevented by 50 mg/l DAP. Therefore it is obvious that diaminopurine does inhibit cell division in these cultures. On media to which 80 mg/l adenine sulfate had been added, the fresh weight of the callus was 82 mg per segment. This weight is not significantly greater than the control weight. In the presence of the adenine, 1 mg/l DAP did not decrease the fresh weight of the callus. However, 5 mg/l DAP gave a definite inhibition although it was less than that which occurred in the absence of adenine. Adenine, therefore, certainly alleviates the DAP inhibition of cell division in the tobacco stem callus.

Reversal of the DAP inhibition was attempted with several other compounds (Table II). Although hypoxanthine and guanine had a slight effect, these two compounds were not nearly as effective as adenine. Arginine and xanthine had little effect, if any, and thymine had no effect. The reversing action of adenine seems to be rather specific. On the control medium, vegetative buds developed from the callus and internal phloem of the tobacco stem pieces(3,4). In the experiment cited in Table I, an average of 3.9 buds per segment were visible at the 39-day reading. The number of buds was reduced to zero when either 1 or 5 mg/l DAP was added to the basal medium. In another experiment (Table II), the number was decreased from 4.5 on the control medium to 1.4 on media containing 0.5 mg/l DAP.

In contrast to the callus growth which seemed little affected, bud formation was promoted by the presence of adenine in the basal medium(3). The number of buds was increased to an average of 6.6 per segment on media containing 80 mg/l adenine sulfate. DAP at a concentration of 1 mg/l reduced this value to 5.1 and at 5 mg/1 to 0.8 bud per segment. Adenine thus alleviates the DAP inhibition of bud formation.

Autoclaving did not alter the effectiveness of the DAP in inhibiting bud formation and callus growth, nor did it affect the reversal by adenine since identical results were obtained when the DAP was Seitz-filtered and added to cooled autoclaved media as when it was added to the media before autoclaving.

Of the other compounds tested for reversing action, only guanine and hypoxanthine showed any activity (Table II). As with callus growth, these two compounds were much less effective than adenine in reversing the DAP inhibition of budding.

Cell division in habituated tobacco callus. The experiments with 2,6-diaminopurine have been extended to physiologically different types of cells. Strain No. 3137 of tobacco callus has been cultured continuously in our laboratories for 4 years and grows well on the same basal medium as is used for the culture of tobacco stem segments(3). Derived from tissues in which an exogenous source of auxin (indole-3-acetic acid or related compounds) was a requisite for growth, this strain may be called habituated since added auxin is not now necessary for its growth. That 2,6-diaminopurine markedly affects cell division in this type of callus is indicated by the following relative increases of fresh weight during a growth period of 28 days: control, 100; 0.1 mg/l DAP, 73; 0.5 mg/l DAP, 49; 1 mg/l DAP, 36; and 5 mg/l DAP, 17. Thus, diaminopurine inhibits cell division in both habituated and non-habituated tissues.

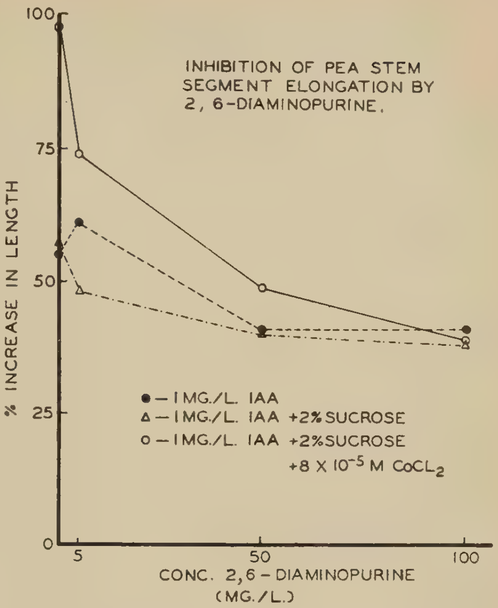


FIG. 1. Inhibition of pea stem segment elongation by 2,6-diaminopurine when added to various solutions of 1 mg/l indole-3-acetic acid (IAA), 2% sucrose, and 8×10^{-5}M $CoCl_2$. Readings made 24 hr after start of test. Original length was 5.3 mm.

Cell enlargement in tobacco stem pith pieces. Pieces of tobacco stem pith when cultured on the basal medium exhibit no cell division but enlarge greatly when supplied with indole-3-acetic acid (IAA)(5,6). In the experiment represented in Table IIIA, the cell enlargement as measured by increases of fresh weight was three times as great on a medium containing 2 mg/l IAA as it was on the control medium. This cell enlargement on media containing IAA was largely prevented by inclusion of 2,6-diaminopurine. DAP at 5 mg/l eliminated most of the enlargement. At concentrations necessary for reversal of the DAP inhibition, adenine itself was somewhat inhibitory. Nevertheless, reversal did occur. Data supporting this conclusion are presented in Table IIIB. The inhibition due to 5 mg/l DAP was prevented by 5 or 10 mg/l adenine. Also guanine • HCl at a level of 20 mg/l apparently reduced the effect of the DAP.

Cell enlargement in etiolated pea stem segments. Elongation of segments from etiolated pea plants may be used as a measure of cell

TABLE III. Inhibition by 2,6-diaminopurine (DAP) of Cell Enlargement in Tobacco Stem Pith Pieces; Reversal with Adenine.*

Addenda						
IAA (mg/l)	DAP (mg/l)	Adenine (mg/l)	No. pieces	Fresh wt/ piece (mg)	Increase fresh wt/ piece (mg)	Relative fresh wt increase
A.						
—	—	—	15	196	65	100
2	—	—	15	327	196	302
2	0.1	—	15	294	163	251
2	1	—	15	311	180	277
2	5	—	15	213	82	126
2	50	—	15	211	80	123
B.						
—	—	—	15	153	42	100
2	—	—	15	257	146	348
2	5	—	15	157	46	109
2	—	5	15	223	112	267
2	—	10	15	234	117	279
2	5	5	15	229	118	281
2	5	10	15	235	124	295
2	5+ (20 mg/l guanine · HCl)		15	193	82	195

* Weighings made 7 days after start of experiment. IAA = indole-3-acetic acid.

enlargement. Alaska No. 323 pea plants were grown in an "*Avena*" darkroom at a temperature of 25°C and relative humidity of about 88 per cent for 6-7 days. At this time sections 5.3 mm long were cut from the third internode just below the apex. Such sections, when floated for 24 hours on a solution of 2% sucrose and 1 mg/l IAA (pH 6.0) increased in length 57% (Fig. 1). Diaminopurine at 50 mg/l reduced the amount of elongation to about 40%. Growth in length was increased from the control value of 57% to a value of 98% of the original length when $CoCl_2$ (8 x 10^{-5}M) was added to the sucrose-IAA medium(7). DAP completely overcame this effect of cobalt and reduced growth to the same level as in the control segments. As may be seen from the dashed curve of Fig. 1, DAP reduced elongation when sucrose was not included in the test solution.

The rise in this curve at 5 mg/l DAP was not observed in subsequent experiments. The reductions in elongation in all treatments were correlated with lower fresh weights. Since the increase in length of the segments is the result of cell elongation, this process apparently is affected by DAP in pea stem segments. The inhibition due to DAP was prevented in part by addition of adenine, adenosine, or adenylic acid to the test solution (Table IV). At concentrations at which adenine prevented the inhibition, it itself was somewhat inhibitory to cell elongation.

Cell enlargement in Avena coleoptile segments. Segments, 5.3 mm in length, were cut from dark-grown (in the "*Avena*" room) oat coleoptiles 72 hours after soaking the seeds. These segments increased 87% in length when floated for 24 hours on a solution containing 2% sucrose and 2 mg/l IAA. The elongation was reduced to 52% when 100 mg/l 2,6-diaminopurine was included in the test medium.

Discussion. On the basis of the results obtained it may be concluded that cell division and cell enlargement in plants, and bud formation (involving both cell division and enlargement) can be inhibited by 2,6-diaminopurine. Furthermore, the inhibition may be almost completely if not entirely prevented by adenine. The results are in general agreement with those obtained with viruses, microorganisms, and animals by other investigators.

What the mechanism of DAP action may be is not known although the present results show that the DAP affects processes essential for both enlargement and division of plant cells. If the inhibitory action is via an interference with nucleic acid metabolism(8), then the participation of nucleic acids in cell enlargement is likely. From results obtained with *Lactobacillus casei,* Balis *et al.*(9) concluded that DAP inhibition is not through pentose nucleic acid but may be through

TABLE IV. Inhibition of Pea Stem Segment Elongation by 2,6-diaminopurine (DAP); Reversal by Adenine, Adenosine, and Adenylic Acid.*

Addenda					
DAP (mg/l)	Adenine (mg/l)	Adenosine (mg/l)	Adenylic acid (mg/l)	% elongation	Relative elongation
—	—	—	—	58	100
50	—	—	—	36	62
—	50	—	—	55	95
—	—	100	—	61	105
—	—	—	100	61	105
50	50	—	—	48	83
50	—	100	—	47	81
50	—	—	100	51	88

* 1 mg/l indole-3-acetic acid and 2% sucrose included in all solutions. Readings made 24 hr after start of test.

desoxypentose nucleic acid, coenzymes, or nucleotides such as adenosinetriphosphate. An effect on the last two types of compounds could interfere with the utilization of energy in plant growth(10,11).

Summary. 1. 2,6-diaminopurine has been shown to inhibit cell division in tobacco stem callus and habituated tobacco callus cultures, to prevent the formation of buds in tobacco stem pieces cultured *in vitro,* to reduce cell enlargement in tobacco pith pieces cultured *in vitro,* and to inhibit elongation of etiolated pea stem and *Avena* coleoptile segments floated on test solutions. 2. Adenine, arginine, guanine, hypoxanthine, and xanthine were tested in attempts to reverse the inhibition by diaminopurine of callus growth and bud formation in tobacco stem segments. Adenine was very effective and slight reversal was obtained with guanine and hypoxanthine. 3. Reversal of the DAP inhibition of tobacco pith cell enlargement was obtained with adenine and guanine, but adenine was more effective. No other compounds were tested on this tissue. 4. Reversal of the diaminopurine inhibition of etiolated pea stem segment elongation was attempted only with adenine, adenosine, and adenylic acid. All three compounds gave partial reversal.

---

1. Hitchings, G. H., Elion, G. B., VanderWerff, H., and Falco, E. A., *J. Biol. Chem.,* 1948, v174, 765.
2. DeRopp R. S., *Can. Res.,* 1951, v11, 663.
3. Skoog, F., and Tsui, C., *Am. J. Botany,* 1948, v35, 782.
4. Sterling, C., *Am. J. Botany,* 1951, v38, 761.
5. Skoog, F., and Tsui, C., unpublished.
6. Jablonski, J. R., Ph.D. Thesis, University of Wisconsin, 1952.
7. Miller, C. O., to be published.
8. Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., and VanderWerff, H., *Ann. N. Y. Acad. Sci.,* 1950, v52, 1318.
9. Balis, M. E., Levin, D. H., and Brown, G. B, *J. Biol. Chem.,* 1952, v196, 729.
10. Albaum, H. G., *Ann. Rev. Plant Physiol.,* 1952, v3, 35.
11. Bonner, J., and Bandurski, R. S., *Ann. Rev. Plant Physiol.,* 1952, v3, 59.

Received April 9, 1953. P.S.E.B.M., 1953, v83.

Sedormid-Induced Porphyria in the Rat.* (20419)

James D. Case, Robert A. Aldrich, and Richard A. Nevé. (Introduced by E. S. West)

From the Departments of Biochemistry and Pediatrics, University of Oregon Medical School, Portland

Duesberg suggested that prolonged ingestion of allylisopropyl-acetyl-carbamide (Sedormid)† may have played a role in the development of acute porphyria in one of his patients(1). Recently, Schmid and Schwartz administered this compound to rabbits and produced a syndrome resembling adult human porphyria(2). The liver of these animals appeared to be one of the principal organs involved.

We have administered Sedormid to another species (rat) and observed a similar effect. This is of particular interest because a vast amount of biochemical information is available on rat liver.

Materials and methods. Female Sprague-Dawley rats weighing approximately 200 g were used. Sedormid was suspended in propylene glycol (50 mg per ml) and given by stomach tube in single daily doses of 200 to 400 mg per kg. The animals were allowed an unlimited intake of food (Purina chow) and water. Daily observations were made of weight, food and water intake, number of fecal pellets, appearance, strength and alertness. Seven control animals received propylene glycol alone by stomach tube in daily doses of 1.2 to 2.0 ml per kg. Six rats received subcutaneous injections of Sedormid in daily doses of 200 to 400 mg per kg. One rat received 100 mg Sedormid per day intraperitoneally. Sedormid was suspended for these parenteral injections in isotonic glycerol containing 0.1% "Tween 80."‡ Twenty-four-hour urine specimens collected in metabolism cages (3) were subjected to 4 separate determinations. Coproporphyrin was estimated by the method of Schwartz *et al.*(4). "Unboiled" uroporphyrin concentration was determined by combining the aqueous phase from the coproporphyrin determination with the 1% sodium acetate washes. This solution was then made to 1.5 N with HCl and the uroporphyrin determined by fluorescence against a coproporphyrin standard.§ An aliquot of the native urine was analyzed for its "boiled" uroporphyrin content after the sample was made to pH 5.0 with buffered acetic acid and boiled for twenty minutes to convert any uroporphyrin precursors. It was then estimated in exactly the same manner as "unboiled" uroporphyrin. Finally the native urine sample was tested for porphobilinogen using the method of Watson and Schwartz(5). The ratio of coproporphyrin isomers I and III was determined when porphyrin excretion was maximum(6). Uroporphyrin methyl ester was crystallized and characterized by its physical properties(7,8).

Results. Table I presents data on urinary porphyrin of normal rats, animals receiving oral propylene glycol alone, and animals receiving parenteral Sedormid injections. Table II shows the quantitative urinary porphyrin values from 9 rats at the height of the Sedormid-induced disorder. The magnitude and variability of the response to the drug are illustrated. Fig. 1 depicts the chemical and clinical course of the Sedormid effect on a typical animal. The large amounts of uroporphyrin which appeared in the rat urine were predominantly in the form of a non-fluorescent precursor which converted to uroporphyrin on boiling. Chromatography and crystallization of this porphyrin as its methyl ester yielded crystals which resembled curved hairs and melted at 260 to 261°C (uncorrected). The isomer

---

*Supported in part by a grant from the Helen Hay Whitney Foundation. Presented in part at annual meeting of Western Society for Clinical Research, Carmel, Calif., Jan. 30, 1953.

† We are indebted to Hoffmann-LaRoche, Nutley, N. J., for generous supply of this compound.

‡ We are indebted to Dr. Ellen Talman, Dept. of Bacteriology, Univ. of Oregon Medical School, for devising and preparing this suspension.

§ All uroporphyrin values reported herein are in terms of coproporphyrin.

TABLE I. Urinary Porphyrins of Normal Rats and Rats Receiving Oral Propylene Glycol or Parenteral Sedormid Alone. 200 g female Sprague-Dawley rats.

Description	No. of determinations	C	U	UB	Daily dosage	Days treated
13 normal rats	59*				—	—
Avg		12.2	5.6	5.4		
Range		3.2–25.8	0.5–13.0	1.6–13.9		
7 rats given oral propylene glycol	7				1.2–2.0 ml	11–21
Avg		10.6	5.8	5.4		
Range		6.9–13.1	4.1–8.8	4.0–8.0		
6 rats given subcut. Sedormid	6				200–400 mg/kg	35–37
Avg		18.3	8.6	8.6		
Range		8.5–24.0	3.0–14.1	3.1–14.7		
1 rat given intraper. Sedormid	5				100 mg/kg	11
Range		12.5–17.7	3.3–5.7	4.6–7.0		

Values expressed as μg of coproporphyrin/24 hr. C = coproporphyrin; U = "unboiled" uroporphyrin, UB = "boiled" uroporphyrin. Porphobilinogen tests all were negative.

* 59 24-hr urine samples from the 13 rats.

TABLE II. Maximum Urinary Porphyrin Values during Oral Sedormid Administration.* 9 rats.

Drug administered, days	C	U	U(B)	P
25	98	42.5	1550	++++
15	150	105	1575	++++
16	93	16.7	116	++++
29			3230	++++
11	189	98.3	1254	++++
20	108	51.3	634	++++
12	13	48	114	++
12	232	168	1130	+++
13	114	100	400	++

* Values expressed as μg of coproporphyrin/24 hr.

C = coproporphyrin; U = "unboiled" uroporphyrin; U(B) = "boiled" uroporphyrin; P = porphobilinogen qualitative test.

ratio of the purified coproporphyrin was 90% type III and 10% type I.

A striking clinical change occurred coincident with the appearance of large amounts of uroporphyrin and porphobilinogen in the urine. The animals showed marked weakness, most evident in the hind legs. Lethargy was also apparent, together with weight loss, decreased intake of food and water, and reduced numbers of fecal pellets. A few rats continued to eat relatively well and maintained their weight. If the drug was continued, progressive paralysis and death in coma ensued. However, if Sedormid administration was stopped, complete clinical and chemical recovery usually took place in 48 to 72 hours. Autopsy of animals dead from Sedormid under a Woods light revealed no red fluorescence in the liver or bile duct. On cut surface the kidney exhibited a brilliant band of red fluorescence at the cortico-medullary junction which was not seen in normal animals.

Discussion. The data in Table I indicate that the amount of coproporphyrin in normal rat urine is relatively constant, with a range in 59 determinations of 3.2 to 25.8 μg per day. Levels of uroporphyrin ranged from 0.5 to 13.9 μg per day, and were essentially unchanged by boiling. This last fact is important because it indicates that no uroporphyrin precursor is present in normal rat urine.

Fig. 1 shows that after Sedormid administration, there is a steady rise of urinary coproporphyrin The levels of both "unboiled" and "boiled" uroporphyrin remain relatively low until a high level of coproporphyrin is reached, during 1 to 2 weeks of drug administration. A sharp increase in uroporphyrin excretion is then observed; simultaneously a strong positive porphobilinogen test appears, and the animal becomes seriously ill. The finding of a non-fluorescent uroporphyrin precursor, as shown by the increase of "boiled" over "unboiled" uroporphyrin, has been observed by others in both human and experimental rabbit porphyria(9,2). The melting point and crystal morphology of the uropor-

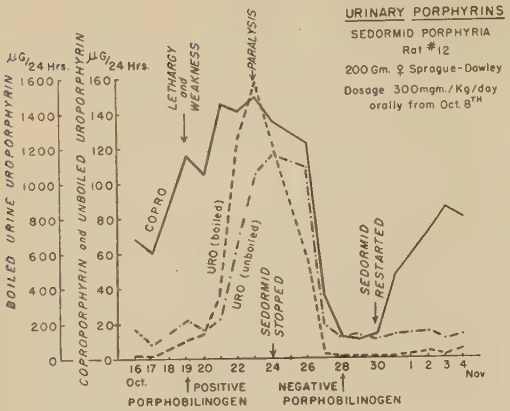


FIG. 1.

phyrin methyl ester suggest that it is probably uroporphyrin type III or the Waldenström uro-type porphyrin.

Subcutaneous injections of Sedormid in dosages that are effective orally caused no disorder of porphyrin excretion (Table I). Propylene glycol given alone by stomach tube likewise produced no change in urinary porphyrins.

The clinical features presented by Sedormid treated animals, the presence of porphobilinogen, increased amounts of predominantly type III coproporphyrin, and enormous amounts of uroporphyrin in the urine very closely approximate the criteria considered diagnostic of porphyria in human adults. The ready availability of extensive biochemical data on rat liver seems to make rats preferable to rabbits for the study of intermediary porphyrin metabolism. Furthermore, the similarity of our findings to those of the University of Minnesota investigators indicates that the effect of Sedormid is not limited to rabbits. The work is being continued.

Summary. Sedormid administered to rats by stomach tube has caused marked increases in urinary uroporphyrin, coproporphyrin, and porphobilinogen, and concurrent clinical changes of paralysis, lethargy and gastrointestinal dysfunction. These findings constitute a syndrome closely resembling human adult porphyria. The disorder thus produced resembles that recently reported in rabbits.

---

1. Duesberg, R., *Münchener Med. Wchschr.*, 1932, v79, 1821.
2. Schmid, R., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 689.
3. Guest, G. M., Brodsky, W. A., and Nelson, N., *Metabolism*, 1952, v1, 89.
4. Schwartz, S., Keprios, M., and Schmid, R., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 463.
5. Watson, C. J., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 393.
6. Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, v168, 133.
7. Watson, C. J., Schwartz, S., and Hawkinson, V., *J. Biol. Chem.*, 1945, v157, 345.
8. Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, 1945, v157, 323.
9. Hawkinson, V. E., and Watson, C. J., *Science*, 1952, v115, 496.

Received April 28, 1953. P.S.E.B.M., 1953, v83.

Rat Plasma Aminopeptidase Activity in Hemorrhagic Shock.* (20420)

THEODORE B. SCHWARTZ† AND FRANK L. ENGEL.
From the Department of Medicine, Duke University School of Medicine, Durham.

As part of an investigation of the biologic significance of peptidases of blood and tissue, plasma peptidase activity was studied in rats subjected to hemorrhagic shock. The striking alterations which appeared are presented in this report.

Materials and methods. Shock was established in rats by bleeding from the cut tail as described previously(1). Serial samples of nonhemolyzed heparinized plasma were obtained by centrifugation of blood removed from

---

* This work was supported by grants from the National Institutes of Health of the U. S. Public Health Service, Duke Research Council and by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under contract.

† Damon Runyon Senior Clinical Research Fellow.

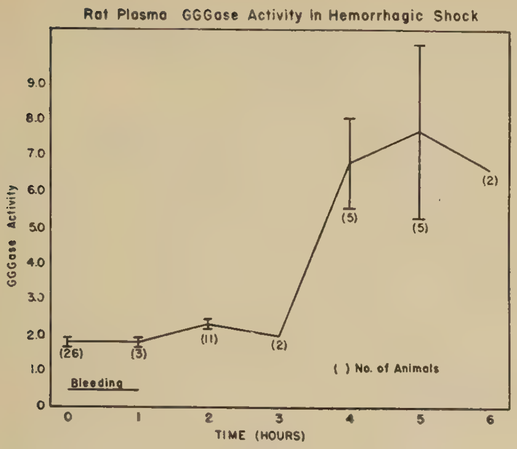


FIG. 1. GGGase activity (see text) at 4 and 5 hr is significantly higher ($p < 0.01$) than that measured at zero, one and 2 hr. Vertical lines represent stand. errors.

the cut tail or, terminally, from the abdominal aorta. Plasma aminopeptidase (GGGase) activity was determined by measuring the rate at which a synthetic substrate, glycylglycylglycine (GGG)‡ was hydrolyzed by rat plasma. The enzyme-substrate mixture, consisting of 0.5 ml of 0.1 M GGG, 0.2 ml plasma, 0.5 ml of 0.02 M veronal buffer (pH 7.8) and 0.8 ml of 0.85% sodium chloride, was shaken constantly in a bath maintained at 38°C. Samples were withdrawn for analysis at one and 2 hours after initiation of enzymic hydrolysis. Measurements were obtained with a photometric technic(2). The mean of the one and 2 hour determinations was used to estimate plasma GGGase activity, defined as the number of μM of GGG hydrolyzed per hour per 0.1 ml of plasma per ml of hydrolysis mixture. Calculations were made on the assumption that there is cleavage of only one peptide bond for each molecule of substrate. This assumption is strengthened by the finding that, after hydrolysis had proceeded for 18 hours, the per cent of substrate split never exceeded 100%. Plasma α-amino nitrogen was determined by the method of Frame, Russell, and Wilhelmi(3).

Results. The effect of the production of hemorrhagic shock in rats on plasma aminopeptidase activity is charted in Fig. 1. No change in GGGase activity was discernible during the first 3 hours after the start of the bleeding procedure. After this period, however, a striking but variable rise appeared and persisted through the second 3-hour interval.

That plasma amino nitrogen increases in shock is well established. In 12 animals subjected to shock, GGGase activity and α-amino nitrogen were determined on the same plasma samples in 29 instances. As indicated in Fig. 2, despite considerable individual variation, the 2 sets of values tended to increase proportionately, so that there was a general positive correlation which is probably significant as suggested by the correlation coefficient of 0.45 with $p < 0.05$.

Discussion. The rise in plasma aminopeptidase during hemorrhagic shock becomes meaningful when viewed in the context of previous studies.

Interest in serum peptidases was stimulated by the search for a burn toxin. Zamecnik, Stephenson, and Cope(4) found that in dogs and calves subjected to burns, there is a rise in aminopeptidase activity in the serum and in the lymph draining the burned area. Beloff and Peters(5) made the complementary observation that proteinase concentration of skin decreases in areas where burns were produced by temperatures which did not inactivate this enzyme. More recently, Stern *et al.*

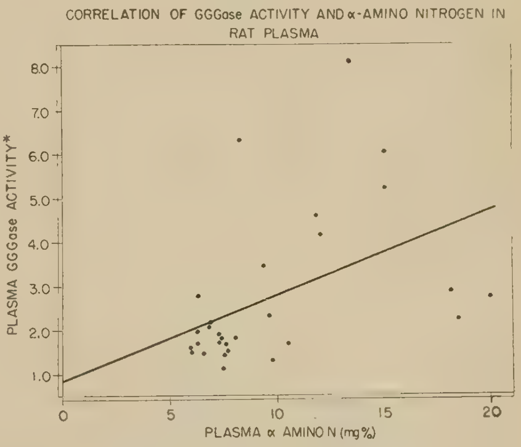


FIG. 2. Positive correlation between GGGase activity and α-amino nitrogen is shown ($r = 0.45$, $p < 0.05$) for 29 plasma samples taken from 12 animals. The linear regression curve has the equation $Y = 0.193X + 0.854$.

* See text.

---

‡Provided through the courtesy of Dr. R. J. Floody, Hoffmann-LaRoche.

(6) found in patients who had suffered fractures, a rise in serum aminopeptidase followed by a fall to normal during convalescence. Several groups of investigators(4,7,8) showed clearly that the mammalian erythrocyte is a rich source of peptidases and that hemolyzed serum is abnormally high in peptidase activity. Smith and associates(9) found significant increases in a variety of serum peptidases following the administration of hemolyzing doses of phenylhydrazine to dogs. In addition, we have noted previously(10) that in certain patients having high plasma peptidase activity, clinical improvement following ACTH or cortisone therapy was paralleled by a fall in plasma peptidase activity to normal values. This observation has been confirmed (11). Thus, it seems clear that tissue destruction may be associated with elevated plasma peptidase activity and that with healing there is a return to normal peptidase levels. This is in accord with the conclusion of Cullen *et al.*(12) that "pathological conditions which are associated with cellular damage result in a high serum peptidase activity, apparently due to a leakage of these enzymes out of the cells."

Among the experimental studies just cited, increased plasma peptidase activity appeared as a consequence of clear-cut cell destruction. In contrast, it is interesting that, in the present investigation, striking elevation in plasma GGGase activity occurred under conditions in which there was no obvious mechanical disruption of cellular elements. Instead, cell damage is attributable to tissue hypoxia. The degree of reversibility of this form of metabolic injury is not well defined but, in a concurrent study(1), it was clearly shown that, when exposed to oxygen, surviving diaphragms of rats subjected to hemorrhagic shock exhibit changes which may be interpreted as evidence of "recovery" from the shocked state.

Summary. When profound shock is produced in rats by severe hemorrhage, a striking rise in plasma aminopeptidase activity occurs. This elevation of plasma GGGase activity is roughly correlated with the increment in plasma amino nitrogen.

The valuable assistance of Mrs. Catherine C. Towbin and Miss Kathleen Hewson is gratefully acknowledged.

---

1. Schwartz, T. B., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 362.

2. Schwartz, T. B., and Engel, F. L., *J. Biol. Chem.*, 1950, v184, 197.

3. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, 1943, v149, 255.

4. Zamecnik, P. C., Stephenson, M. L., and Cope, O., *J. Biol. Chem.*, 1945, v158, 135.

5. Beloff, A., and Peters, R. A., *J. Physiol.*, 1945, v103, 461.

6. Stern, K., Cullen, A. M., and Barber, V. T., *J. Clin. Invest.*, 1949, v28, 419.

7. Johansen, A., and Thygesen, J. E., *Compt.-rend. trav. Lab. Carlsberg, Ser chim.*, 1948, v26, No. 14.

8. Schwartz, T. B., and Engel, F. L., *J. Biol. Chem.*, 1949, v180, 1047.

9. Smith, E. L., Cartwright, G. E., Tyler, F. H., and Wintrobe, M. M., *J. Biol. Chem.*, 1950, v185, 59.

10. Schwartz, T. B., and Engel, F. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 82.

11. Johansen, A., and Thygesen, J. E., *Scand. J. Clin. Lab. Invest.*, 1951, v3, 66.

12. Cullen, A. M., Barber, V. T., Birmingham, M. K., and Stern, K., *Canad. J. Med. Sci.*, 1951, v29, 114.

Received May 4, 1953. P.S.E.B.M., 1953, v83.

Placental and Mammary Transfer of Vitamin A and Carotene by Beef Cows. (20421)

F. H. BAKER, ROBERT MACVICAR, L. S. POPE, AND C. K. WHITEHAIR.*

From the Departments of Animal Husbandry and Agricultural Chemistry Research, Oklahoma A. and M. College, Stillwater, Okla.

The carotene and vit. A nutrition of cattle is greatly influenced by their ability to store large quantities of this vitamin while grazing green pasture. The major site of carotene and vit. A storage, according to Guilbert and Hart(1), is in the liver. It has been reported by Speilman *et al.*(2) that supplementation of dairy cow rations with high levels of vit. A during the last 30 days of gestation increased plasma levels and liver stores of vit. A in newborn calves. Fountain *et al.*(3) observed that calves from dairy cows maintained on green pasture during late gestation had significantly increased plasma vit. A levels at birth. Eaton and coworkers(4) reported that the vit. A content of the liver was significantly higher at birth and at 30 days of age in pigs and lambs whose dams received supplementary vit. A during late gestation. Henry and associates(5) have shown that the vit. A content of the milk of rats and the liver stores of the nursing offspring are influenced to a greater degree by the amount of vit. A in the diet of the mothers during lactation than by their liver reserves.

As a part of a long-time study of carotene metabolism in beef cattle, investigations have been made of the effect of level of carotene intake on liver stores of cows at parturition and of their newborn calves. Also included in the study were the effects of maternal liver stores and level of carotene intake on the vit. A content of the milk and on the blood and liver levels of the calves at 3 months of age.

Experimental. Ten 2-year-old Hereford cows in the second and third month of pregnancy were selected in March, 1952, for the experiment. During gestation, each of the cows was fed the following low-carotene ration daily: cottonseed meal, 2½ lb; ground milo, 1 lb; dried beet pulp, 2 lb; cottonseed hulls, 2 lb; and wheat straw or dry weathered range grass, *ad libitum*. During lactation, the low-carotene ration for each cow was composed of cottonseed meal, 3 lb; ground milo, 4 lb; and wheat straw, *ad libitum*. The cattle had access to a mineral mixture† and salt at all times. The basal ration was calculated to meet the recommended allowances of the National Research Council for all essential nutrients for beef cattle with the exception of carotene. During the experiment, the cows were maintained on concrete floors and had no access to carotene-containing feeds. At the beginning of the experiment, blood samples and liver biopsy samples were collected by the technic of Whitehair and associates(6). Assignment of the cows to the various treatments was based on liver stores of carotene and vit. A. To study the ability of cows to mobilize liver stores during lactation, high liver levels were desirable; consequently those animals with the highest liver stores were assigned Lots III and IV. The treatments and number of cows in each were: Lot I, no supplemental carotene, 2; Lot II, supplemental carotene during lactation, 3; Lot III, supplemental carotene during gestation, 3; Lot IV, supplemental carotene during gestation and lactation, 2. The levels of carotene administered were in accordance with the National Research Council's recommended daily allowances for beef cows of 60 μg of carotene/lb body weight during gestation and 330 μg/lb during lactation. A crude carotene concentrate‡ derived from alfalfa and containing 6 mg of carotene per g was administered in gelatin capsules to meet the specified allowances. The capsules were administered twice weekly during gestation and 3 times weekly during lactation. In addition to the liver and blood samples collected

---

* Present address: College of Veterinary Medicine, University of Illinois, Urbana.

† The mineral mixture contained equal parts of ground limestone, steamed bone meal and salt.

‡ The carotene concentrate was supplied by the Chlorophyll Chemical Corp., McAllen, Texas.

TABLE I. Average Vit. A and Carotene Content of Livers of Beef Cows, and Vit. A Content of Livers of Their Calves (μg/g dry matter).

	Lot No.	6 mo prepartum	At parturition	3 mo post partum
		Cows		
Liver vit. A	I	68.9	5.7	1.7
	II	70.6	9.4	18.9
	III	122.6	20.6	7.4
	IV	92.2	14.3	23.4
" carotene	I	4.0	2.3	1.1
	II	4.7	2.7	3.7
	III	5.9	2.6	2.4
	IV	3.9	3.5	5.2
		Calves		
" vit. A	I		4.2	1.9
	II		3.4	14.2
	III		2.6	5.1
	IV		3.0	22.3

at the beginning of the experiment, liver samples were taken from the cows and their calves immediately after parturition and at the end of 3 months of lactation. Blood samples were collected from the cows approximately 3 months before parturition. Additional blood samples were taken from the cows and their calves immediately after parturition and at 2 weeks, one month, 2 months, and 3 months post partum. Colostrum samples were collected after parturition and milk samples were obtained each time a blood sample was taken from the cow during lactation. The calves were separated from the cows 4 hours before milk samples were collected. One quarter of each cow's udder was milked completely to obtain a representative milk sample. The blood, milk and liver samples were analyzed for carotene and vit. A. Liver samples were analyzed according to the method of Gallup and Hoefer(7), plasma by the method of Kimble(8), and milk by the method of Lesher *et al.*(9).

Results and discussion. The average liver levels of vit. A and carotene for the 4 lots of cows and calves are presented in Table I. Plasma and milk levels of vit. A and carotene for the cows and calves are shown in Table II. Negligible amounts of carotene were found in the milk, or in the blood and liver of the calves; consequently, the carotene contents of these samples are not given.

Examination of the liver composition of the cows at the beginning of the experimental period and at parturition shows that the amount of carotene administered to the cows of Lots III and IV was insufficient to maintain liver stores of vit. A at their initial level. Cows receiving carotene supplementation during gestation had about twice as much vit. A in their livers at parturition as those maintained on the depletion ration. The amount of carotene provided the cows of Lots II and IV during lactation resulted in an increase in liver stores of vit. A despite milk production.

TABLE II. Average Vit. A and Carotene Content of Plasma and Milk of Beef Cows and Vit. A Content of Plasma of Their Calves (μg/100 ml).

	Lot No.	6 mo prepartum	3 mo prepartum	At parturition	Lactation			
					2 wk	1 mo	2 mo	3 mo
			Cows					
Plasma vit. A	I	24.9	8.3	11.3	11.1	10.1	14.2	13.3
	II	22.5	8.7	15.1	18.3	18.7	15.7	24.2
	III	22.3	10.1	16.3	20.5	14.6	15.0	15.1
	IV	21.1	14.2	16.3	17.7	21.4	21.8	23.5
" carotene	I	40.3	11.6	11.8	8.8	6.0	5.4	12.0
	II	45.8	12.9	10.3	52.6	34.5	31.2	43.0
	III	46.6	31.1	23.4	15.7	13.1	11.1	13.0
	IV	35.2	25.8	24.5	87.8	54.8	36.8	46.7
Milk and colostrum vit. A	I			45.6	4.9	4.1	3.3	2.8
	II			72.6	12.6	4.6	2.3	5.4
	III			163.9	4.4	2.5	3.6	3.2
	IV			90.6	7.7	4.0	7.7	4.5
			Calves					
Plasma vit. A	I			3.1	6.2	6.0	6.0	4.1
	II			1.3	9.3	5.7	8.1	9.7
	III			3.6	6.8	5.0	6.3	6.7
	IV			4.1	14.4	6.2	8.0	11.9

If maintenance of liver stores of vit. A is used as a criterion for determining the requirement of this nutrient, 60 μg per lb of body weight appears inadequate for the cow during gestation. However, no symptoms indicative of avitaminosis A were observed in any of the cows at any time during the experimental period. It is recognized that the stores of vit. A at the beginning of the experimental period were high, reflecting a high intake of carotene during the previous summer, and that failure to maintain them is not necessarily indicative of an inadequate carotene intake.

Liver stores of vit. A in newborn calves were not related to the carotene intake during gestation or liver stores of the cows at parturition. It should be noted that the amounts of carotene administered in these studies are distinctly less than those fed by Fountain *et al.* (3) or the vit. A fed by Speilman *et al.*(2) or by Eaton *et al.*(4). This may account for the failure to observe substantial placental transfer of vit. A. These findings are in agreement with the report of Thomas *et al.* (10) who found only small amounts of vit. A in the livers of newborn kids whose dams had been fed natural rations containing the recommended allowance of carotene for the goat. The vit. A content of the livers of the calves at 3 months of age appeared to be affected more by the carotene intake of the dam during lactation than by her liver stores at parturition. The higher liver stores of the cows are reflected in an increase in the liver vit. A of the calves at 3 months of age. Examination of the plasma vit. A levels of the calves reveals a similar trend. It will be noted that there was an actual decrease in the vit. A stores in the livers of calves of Lot I from birth to 3 months of age.

The plasma carotene levels of the cows reflected carotene supplementation, but in no case do the average values approach those commonly encountered when cattle are grazing winter range, or consuming good quality roughage(11). At the beginning of the experimental period, plasma vit. A content was normal. By mid-gestation, all animals showed a decline to levels usually considered indicative of a low plane of carotene or vit. A nutrition. Following parturition, the animals receiving supplemental carotene showed normal values for plasma vit. A, a finding consistent with the increase in liver vit. A stores in these animals.

The vit. A content of colostrum was somewhat higher for cows receiving supplemental carotene during gestation than from those that did not. Substantial amounts of this vitamin were found in the colostrum of cows receiving the low-carotene ration. Evidently the cow is able to mobilize her liver reserves to produce a colostrum relatively high in vit. A. As has been previously reported(12), the vit. A content of the milk rapidly declined and remained fairly constant during the second and third months of lactation. Milk vit. A was generally related to carotene supplementation during the lactation period; the effect of supplementation during gestation and liver vit. A stores at parturition was less apparent.

One calf in Lot I developed symptoms of a vit. A deficiency at about 3 months of age. After the termination of the experimental period, this calf responded to the administration of 480,000 I.U. of vit. A. None of the cows in this experiment showed symptoms of vit. A deficiency at any time.

Summary. Beef cows receiving a carotene allowance equivalent to 60 μg per pound of body weight daily were unable to maintain liver stores or plasma vit. A levels during the last 6 months of gestation. When the carotene allowance was increased to 333 μg during lactation, liver stores and plasma vit. A were increased. Vit. A in the plasma and liver of the calf was closely associated with the carotene intake of the dam during lactation, but was also influenced by the liver stores of the cow at parturition.

---

1. Guilbert, H. R., and Hart, G. H., *J. Nutrition*, 1934, v8, 25.

2. Speilman, A. A., Eaton, H. D., Matterson, L. D., and Slate, R. J., *J. Dairy Sci.*, 1948, v31, 670.

3. Fountain, F. C., Parish, D. B., Atkeson, F. W., and Hughes, J. S., *J. Animal Sci.*, 1948, v7, 525.

4. Eaton, H. D., Christian, J. A., Daugherty, F. C., Speilman, A. A., and Matterson, L. D., *J. Animal Sci.*, 1949, v8, 224.

5. Henry, K. M., Kon, S. K., Mawson, E. H., Stanier, J. E., and Thompson, S. Y., *British J.*

Nutrition, 1949, v3, 301.

6. Whitehair, C. K., Peterson, D. R., Van Arsdell, W. J., and Thomas, O. O., *J. Am. Vet. Med. Assn.,* 1952, vCXXI, 285.

7. Gallup, W. D., and Hoefer, J. A., *Anal. Ed. Ind. Eng. Chem.,* 1946, v18, 288.

8. Kimble, M. S., *J. Lab. Clin. Med.,* 1939, v24, 1055.

9. Lesher, M., Brody, J. K., Williams, H. H., and Macy, I. G., *Am. J. Dis. Child.,* 1945, v70, 182.

10. Thomas, J. W., Loosli, J. K., and Williams, J. P., *J. Animal Sci.,* 1947, v6, 141.

11. Long, R. A., Van Arsdell, W. J., MacVicar, Robert, and Ross, O. B., *Okla. Agri. Exp. Sta. Tech. Bull.* No. T-43, 1952.

12. Van Arsdell, W. J., Ross, O. B., and MacVicar, R. W., *J. Animal Sci.,* 1950, v9, 545.

Received May 7, 1953. P.S.E.B.M., 1953, v83.

Chromatographic Separation of the Plasma Lipids.* (20422)

DOROTHY L. FILLERUP AND JAMES F. MEAD.

From the Atomic Energy Project, School of Medicine, University of California, Los Angeles.

Although technics for the analysis of the various components of the plasma lipids are available, methods for their separation are neither simple nor quantitative. Bloor's method(1) of phospholipid separation has been the subject of considerable discussion (2-8) with general agreement that not all the phospholipid is precipitated by acetone-magnesium chloride(3,4,6,9,10) while significant amounts of other water soluble materials such as urea, creatinine, amino acids and Na, K, and Cl salts generally accompany it as impurities(11-13). Resolution in petroleum ether of the alcohol-ether extract may leave as much as 11.8% residue undissolved(13). The separation of the crude mixture into saponifiable and non-saponifiable fractions involves the destruction of both triglycerides and sterol ester fractions and thus precludes their separate analysis. Indeed, the separation and analysis of the sterol and sterol ester fractions has been the subject of considerable investigation, with the resultant disclosure of several procedures(14-21). The more successful of these methods have made use of the technic of chromatography. Trappe(20) investigated the use of alumina and later of silicic acid to separate glycerides from cholesterol esters, whereas Hess(14) used alumina for the separation of sterols and sterol esters. We were unable to repeat the work of Hess, who reported the elution of sterol ester with 10% ether in petroleum ether and of sterols with 10% alcohol in petroleum ether. In our hands both fractions appeared together in the eluate with the alcoholic mixture. Part of the difficulty may lie in the well-known activity of alumina in altering the adsorbed lipid, for example, by hydrolysis of fatty esters(2,20). Silicic acid seemed most suitable for the separation since it is not known to alter the adsorbed materials and shows no reaction with the solvents. Borgstrom(2) used this material to separate phospholipids or cholesterol palmitate from a mixture of stearic acid and glycerides. The last 2 substances could then be separated by extraction of their alkaline alcohol solution with petroleum ether. However, when mono- or diglycerides were present they were only partly extracted and it was necessary to use an ion exchange resin (IRA-400), to effect the separation.

With these considerations in mind, the complete separation of the plasma lipids was attempted with only one column and alterations in the eluting mixture. Regular changes in the eluant were not attempted since it was thought that some advantage could be gained by continuing the elution with a particular solvent mixture until little or no material appeared in the eluate. In this manner, sub-

---

* This paper is based on work performed under contract between the Atomic Energy Commission and the University of California at Los Angeles.

TABLE I. Chromatographic Separation of an Artificial Lipid Mixture.

Fraction (in order of elution)	Compounds used*	Solvent mixture eluting fraction	Column vol of eluate	% recovery
1. Sterol ester	Cholsterol palmitate, cholesterol oleate	1% ether in petroleum ether	20	98.4–100
2. Triglyceride	Tripalmitin, triolein	4% ether† in petroleum ether	20	96 –100
3. Sterol	Cholesterol	10% ether in petroleum ether	15	99 –100
4. Acid	Stearic, oleic	50% ether in petroleum ether	15	97
5. Phospholipid	Lecithin	25% methanol in ether	15	100

* Sterol esters were purchased from Dougherty Chemical Co., the tripalmitin and oleic acid were prepared and purified by Drs. Nevenzel and Howton of this laboratory. Triolein was purchased from Matheson Co.; cholesterol and stearic acid purchased from Eastman Kodak Co., and lecithin from Bios Laboratories.

† Mallinckrodt analytical grade ether was shaken with aqueous ferrous sulfate, dried over anhydrous magnesium sulfate and calcium chloride and redistilled before use.

fractions of the main lipid classes were obtained which will be examined in the future. Artificial mixtures of lipids were studied before applying the technic to the separation of the plasma lipids. As a preliminary application of the method a spectrophotometrically determinable fatty acid, β-eleostearic(22), was fed as the methyl ester, and its distribution among the plasma lipids was investigated. A trial of the different methods of extraction of plasma lipids revealed that in our hands a modification of the method of Delsal(23) gives the highest and most consistent results. This method was therefore adopted for all plasma lipid extractions.

Materials and methods. All solvents were analytical grade when purchased and were redistilled before use. Data on the purification or preparation of other materials are given in the body of the following section.

Total plasma lipid extraction. Blood is removed from the anticubital vein of an adult human and is immediately placed in a 15 ml centrifuge tube containing a few grains of heparin-sodium. This anticoagulant is used in preference to oxalate or citrate since the last two are reported to lead to erroneous values for lipids(9,24-27). The tube is shaken gently to mix the contents and is centrifuged for 15 minutes at 1000 g. To 2 ml of plasma is added, with vigorous stirring, 30 ml of a 4:1 mixture of methylal:methanol (23). After 30 minutes, the precipitate is centrifuged down and the supernatant solution is evaporated to dryness under nitrogen at reduced pressure and room temperature(4,5, 9,28). The residue is extracted with petroleum ether and this extract is evaporated to dryness in the same manner; the residue is taken as the total lipid. The method appears superior because of its reproducibility and high yields. An average of 752.7 mg % total lipid can be compared with that of 545 mg % obtained by the method of Bloor(29) with samples of the same plasma.

Chromatography. For about 50 mg of lipid, 13 of silicic acid was packed into a tube of 2 cm diameter to a height of 7 cm. The column was washed under reduced pressure with 3 column volumes (one volume = about 14 ml) of absolute methanol, followed by equal volumes of acetone; anhydrous, peroxide-free ether; and petroleum ether (b.r. 60-70°). Pure lipids were added to the column in petroleum ether singly, in pairs, or, finally, as a complete mixture, and were eluted from the column with various ether-petroleum ether mixtures. A particular solvent mixture was continued until evaporation of the eluate showed it to contain little or no lipid and it became apparent that a more polar mixture would be necessary to elute the next fraction.

Results. Separation of artificial mixtures. Table I gives the composition of the artificial mixture and the solvent system required to elute each fraction. The average percentage

TABLE II. Chromatographic Separation of Plasma Lipids; 7 Determinations. (Values given as mg % of plasma.)

Total lipid	Sterol ester	Neutral fat	Sterol	Phospholipid	Total recovery, %
1047.5	30.0	477.5	70.0	455.0	98.6
953.3	86.7	260.0	110.0	446.7	94.8
786.7	73.3	250.0	113.3	316.6	95.7
750.0	44.0	288.0	96.0	312.0	98.7
737.0	24.3	370.0	77.0	233.0	95.6
620.0	35.0	293.0	62.5	242.0	102.0
572.5	41.3	278.8	167.5	75.0	98.3

recovery, as judged by the weight of the fraction and qualitative† as to its nature are also given.

Separation of plasma lipids. When plasma lipids from various subjects, extracted as described above, were subjected to the same treatment, the data shown in Table II were obtained.

Transportation of β-eleostearic acid in blood. Approximately 5 g of methyl β-eleostearate was fed to 2 normal adults under conditions previously described(22). After 4 hours a sample of blood was drawn and analyzed as described above. Each fraction was analyzed spectrophotometrically for β-eleostearate as previously described(22). In one case a total of 1.90 mg of β-eleostearate was present in the plasma sample used. Of this amount 79% was found in the first 10 volumes of the 2% ether eluate. No eleostearate was found in any but the neutral fat fraction. In previous determinations in which methyl β-eleostearate was added to the serum before extraction, the eleostearate was recovered totally in the first 10 volumes of 4% ether in petroleum ether.

Discussion. Trappe(20), Hess(14), and Borgstrom(2) showed that a partial separation of the plasma lipids could be achieved with a chromatographic technic. It remained only to simplify and generalize the method so that with one absorbent and a simple series of solvents all the major classes of lipids could be separated.

It should be emphasized that the technic is not intended as a quantitative determination of the plasma lipids; however, the partition may be even more sensitive than is claimed since some separation of subfractions within the major classes was observed. Nevertheless, the procedure has already found several applications. The easily traceable β-eleostearic acid appeared only in the triglyceride fraction after its ingestion as the methyl ester. Since Borgstrom(30) has reported that ethyl oleate appeared in the blood of rats not as the ethyl ester but as the triglyceride, we have assumed, in this preliminary experiment, that eleostearic acid also is transported as the triglyceride, and not as the methyl ester, which might also appear in this fraction. Continuation of this research with the aid of these technics may reveal the mode of transport of other types of fatty acids under a variety of conditions.

Summary. By chromatography on a silicic acid column, artificial lipid mixtures and plasma lipids have been separated into fractions composed mainly of the following groups: sterol esters, triglycerides, sterols, fatty acids and phospholipids. By this technic eleostearic acid is found exclusively in the triglyceride fraction after having been fed as the methyl ester.

---

† The Liebermann-Burchard test for sterols was positive only in fractions 1 and 3.

1. Bloor, W. R., *J. Biol. Chem.*, 1929, v82, 273.
2. Borgstrom, B., a) *Acta Physiol. Scand.*, 1952, v25, 101; b) *ibid.*, 111.
3. Boyd, E. M., *J. Biol. Chem.*, 1931, v91, 1.
4. Ellis, G., and Maynard, L., *J. Biol. Chem.*, 1937, v118, 701.
5. Erickson, B., Avrin, I., Teague, D., and Williams, H., *J. Biol. Chem.*, 1940, v135, 671.
6. Favarger, P., *Helv. Physiol. Pharmacol. Acta*, 1949, v7, 371.
7. Marenzi, A. D., and Cardini, C. E., *J. Biol. Chem.*, 1943, v147, 371.
8. Sinclair, R., and Dolan, M., *J. Biol. Chem.*, 1942, v142, 659.
9. Kirk, E., Page, I., and Van Slyke, D., *J. Biol.*

Chem., 1934, v106, 203.

10. Page, I., Kirk, E., Lewis, W., Jr., Thompson, W., Van Slyke, D., *J. Biol. Chem.*, 1935, v111, 613.

11. Christensen, H. N., *J. Biol. Chem.*, 1939, v129, 531.

12. Folch, J., and Van Slyke, D., *J. Biol. Chem.*, 1939, v129, 539.

13. Pernokis, E., Freeland, M., and Kraus, I., *J. Lab. Clin. Med.*, 1941, v26, 1978.

14. Hess, W. C., *J. Lab. Clin. Med.*, 1947, v32, 1163.

15. Kingsley, G. R., and Schaffert, R. R., *J. Biol. Chem.*, 1949, v180, 315.

16. Koehler, A., and Hill, E., *J. Biol. Chem.*, 1949, v179, 1.

17. Schoenheimer, R., and Sperry, W., *J. Biol. Chem.*, 1934, v106, 745.

18. Selbach, H., and Trappe, W., *Arch. Phychiat. Nervenheilk*, 1944, v117, 541.

19. Smith, R., and Marble, A., *J. Biol. Chem.*, 1937, v117, 673.

20. Trappe, W., a) *Z. Physiol. Chem.*, 1942, v273, 177; b) *Biochem. Z.*, 1940, v306, 316; c) *Biochem. Z.*, 1941, v307, 97.

21. Yasuda, M., *J. Biochem.* (Japan), 1936, v24, 443.

22. a) Mead, J. F., Fillerup, D. L., Decker, A. B., and Bennett, L. R., *J. Nutrition*, 1952, v46, 499; b) Fillerup, D. L., and Mead, J. F., UCLA-148.

23. Delsal, J. L., *Bull. soc. chim. biol.*, 1944, v26. 99.

24. Gardner, J., Gainsborough, H., and Murray, R., *Biochem. J.*, 1938, v32, 1457.

25. Man, E., and Gildea, E., *J. Biol. Chem.*, 1932, v99, 43.

26. Schmidt, L. H., *J. Biol. Chem.*, 1935, v109, 449.

27. Shope, R. E., *J. Biol. Chem.*, 1928, v80, 125.

28. Man, E. B., *J. Biol. Chem.*, 1937, v117, 183.

29. Bloor, W. R., *J. Biol. Chem.*, 1928, v77, 53.

30. Borgstrom, B., *Acta Physiol. Scand.*, 1952, v25, 322.

Received May 18, 1953. P.S.E.B.M., 1953, v83.

Method for Establishing Human Cells in Culture: Susceptibility to Poliomyelitis after Prolonged Cultivation.* (20423)

H. E. SWIM AND R. F. PARKER.

From the Department of Microbiology, School of Medicine, Western Reserve University, Cleveland, O.

The application of tissue culture technics to the study of mammalian viruses has received renewed impetus since Enders and his associates showed that the viruses of poliomyelitis would multiply in cultures of human tissues (1). Quantitative studies have been greatly restricted by the existing tissue culture technics for virus propagation, which usually depend on the explantation of fresh tissue for each virus passage. It would obviously be of great value, therefore, to have established cell strains susceptible to viral infection. Of the established cell cultures, the "L" strain (Earle) has been shown to be susceptible to infection with the virus of Rous sarcoma(2), and to pseudo-rabies and herpes simplex(3). Recently, Scherer, Syverton, and Gey(4) have described a strain of cells susceptible to poliomyelitis. The technics described by Earle for the initiation of cell strains on glass or cellophane surfaces have not been widely used. The purpose of this communication is to describe a simple technic for establishing human cells in mass culture and to show how such cell strains may be maintained. These cultured cells appear to retain their initial susceptibility to viral infection.

Methods. The standard technic for cultivation of tissue in roller tubes has been employed. The lower two-thirds of an 18 x 150 mm culture tube is coated with 0.05 ml chicken plasma. The desired number of tissue fragments are placed on the side of the tube and 0.05 ml chick embryo extract (EE 50) is added and mixed with the plasma. The plasma-extract mixture is then rolled over the surface of the tube, allowed to drain for a few seconds, and the excess is removed, leaving the tissue embedded in a very thin clot. The tube is allowed to stand for 5-30 minutes and

---

* Aided by a grant from The National Foundation for Infantile Paralysis.

then 3 ml of nutrient fluid consisting of 85% Fischer's V-614 solution(5), 10% horse serum and 5% EE 50 are added and the tube stoppered and incubated at 37°C. The nutrient fluid is changed at intervals determined by the decrease in pH from the initial value of 7.6 to approximately 7.0, usually every 2 or 3 days. After one to 10 days, new growth appears in the clot around the explanted fragments. The time depends on the type of tissue and its previous treatment. When growth is well established, the original explants are dislodged and removed with the fluid when the latter is changed, usually within 3 to 4 weeks. By this time, the surface of the tube is covered with a thin sheet of cells. Where lysis of the plasma coagulum has occurred, cells grow freely over the glass. The initial cell population is subcultured by dislodging the cells with a platinum wire bent to fit the curvature of the tube. This procedure yields a suspension containing large masses of cells. These cell masses are largely broken up by drawing the fluid into a serologic pipette and then forcefully expelling it. The pooled suspension from several tubes is centrifuged at 175 g for 10 minutes and the supernatant is discarded. The cells are suspended in a small volume of EE 50 and added to tubes containing chicken plasma in the manner already described. Microscopic examination reveals that single cells and small clumps of cells were carried over and are now embedded in the very thin plasma coagulum lining the culture tube. After the first hour of incubation, the cells have assumed their characteristic appearance and can be seen migrating from the small cell masses. Multiplication begins promptly. Cells which have been subcultured in this manner for 4 to 6 months continue to grow well.

Examination of the original culture tube reveals that many individual cells and small sheets of cells remain on the inner surface. If nutrient fluid is added to the tube, these cells will multiply, and within a short period of time the tube is again lined with cells. These cells, which have grown directly on the glass, can be dislodged as already described and further treated by repeated expulsion from a pipette, or by vigorous shaking, to yield a suspension that contains primarily single cells or small masses (2-12 cells) with an occasional larger fragment. These suspensions may be centrifuged and the cells resuspended in EE 50 and planted in a thin plasma coagulum, or the cells may be planted directly on glass, as described by Shannon and Earle(6). In the latter case, there is a longer interval between planting and the initiation of growth; also, many of the larger cell clumps do not adhere to the glass and are lost when the fluid is changed. After each successive harvest, a sufficient number of cells are left behind on the glass to restore the cell population. In this way, a *mother* tube may serve as a source of cells over an indefinite period. In our experience, the effect of repeated harvesting from a single tube has been to increase the tendency of cells to grow from isolated cells on the tube wall, or from increasingly smaller clumps, with the ultimate effect of reducing the size of the clumps in the cell harvest. This factor influences the efficiency of transplantation of cells to glass, as has been noted by Earle, Waltz, and Shannon(7).

Results. Skin-muscle cells of human embryonic origin which were cultivated for 6 months in the manner described were tested for their susceptibility to the cytopathogenic effect of the MEF_1 strain of poliomyelitis virus. The cells obtained from 6 mother tubes were suspended in 25 ml of nutrient fluid. After removing the larger cell clumps from the suspension, it was dispensed in 1 ml volumes into 13 x 150 mm tubes. The tubes were stoppered, inclined at an angle of approximately 5°, and incubated in stationary racks. After 3 days, when cell growth was well established, the nutrient fluid was replaced with Mixture 199 of Morgan, Morton, and Parker(8), and 0.1 ml of the appropriate dilution of virus suspension was added to each tube. The viral suspension used in this experiment consisted of the fluid phase from a culture of MEF_1 poliomyelitis virus(9) in uterine tissue. The tubes were placed in the roller device and incubated at 37°C. After 3 days the cultures were examined for evidence of cytopathogenic effects. The results are recorded in Table I, and indicate that a very small amount of virus is capable of in-

TABLE I. Susceptibility of Human Embryonic Skin-Muscle Cultures to the MEF$_1$ Strain of Poliomyelitis.

Virus dilution (log)	0	1	2	3	4	5	6	No virus
Results	2/2*	2/2	2/2	3/3	3/3	2/3	1/3	0/3

* Denominator represents number of tubes inoculated with 0.1 ml virus suspension; numerator, the cultures showing degeneration in 3 days.

ducing cytopathogenic effects in a cultured strain of skin-muscle fibroblasts.

The thin clot technic has been successful with all the tissues so far studied, including adult human uterus and testicle, and embryonic skin-muscle, liver, lung and kidney. It appears, however, that for the optimum growth of each cell strain that may be studied, the nutritional requirements, the pH range and other factors will need to be established. Thus, for example, embryonic skin-muscle fibroblasts seem to be more easily maintained in roller tubes than in stationary cultures, whereas testicular and uterine cells may be maintained under either condition. Also, skin fibroblasts appear to maintain undiminished vigor in nutrient fluid containing chick embryo extract, whereas uterine fibroblasts seem to do better in beef embryo extract. On the other hand, it appears that while fibroblasts of testicular origin can be cultured initially in fluid containing chick embryo extract, their rate of growth eventually declines and can be restored to normal by substitution of beef embryo extract.

Summary. A technic is described by which human tissues may be established readily *in vitro* either on glass or in thin plasma clots. Susceptibility to infection with the MEF$_1$ strain of poliomyelitis is retained after prolonged cultivation.

1. Enders, J. F., Weller, T. H., and Robbins, F. C., *Science*, 1949, v109, 85.
2. Sanford, K. K., Likely, G. D., Bryan, W. R., and Earle, W. R., *J. Nat. Cancer Inst.*, 1952, v12, 1317.
3. Scherer, W. F., *Am. J. Path.*, 1953, v29, 113.
4. Scherer, W. F., Syverton, J. T., and Gey, G. O., *Fed. Proc.*, 1953, v12, 457.
5. Fischer, A., and Astrup, T., Proc. Soc. Exp. Biol. and Med., 1948, v67, 40.
6. Shannon, J. E., Jr., and Earle, W. R., *J. Nat. Cancer Inst.*, 1951, v12, 155.
7. Earle, W. R., Waltz, H. K., and Shannon, J. E., Jr., *J. Nat. Cancer Inst.*, 1951, v12, 133.
8. Morgan, J. F., Morton, H. J., and Parker, R. C., Proc. Soc. Exp. Biol. and Med., 1950, v73, 1.
9. Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *Science*, 1943, v98, 452.

Received May 25, 1953. P.S.E.B.M., 1953, v83.

L Type Cultures Isolated from Streptococci.*† (20424)

L. Dienes.

From the Department of Bacteriology, Massachusetts General Hospital and the Robert W. Lovett Memorial, Harvard Medical School, Boston, Mass.

Pleuropneumonia-like colonies and larger colonies of similar structure corresponding to L type colonies of various bacteria grow usually in cultures made from the human mouth, especially from tooth scrapings, if bacterial growth is inhibited by penicillin. The observations to be described originated from an effort to determine the origin of these colonies and their eventual connection with bacteria. Numerous colonies of various types of bacteria were picked from anaerobic mouth cultures and were tested for the production of L forms. None of the bacteria produced L type colonies similar to those of the oral pleuropneumonia-like organisms, but larger colonies similar to those seen in the plates directly inoculated with tooth scrapings were produced by an alpha-hemolytic streptococcus (strain 40). Another streptococcus strain with similar properties (Ch 45) was isolated later from the tonsillar exudate of a patient with a sore throat.

---

* Expenses of this investigation defrayed in part by grants from the Commonwealth Fund and the U. S. Public Health Service.

† This is publication No. 147 of the Robert W. Lovett Memorial Foundation for the study of Crippling Disease.

Streptococcus *strain 40* grows on horse blood agar plates in small alpha hemolytic colonies consisting of pairs and short chains. Broth cultures consist of long chains. The coccus is not bile-soluble and is not heat-resistant. It is moderately sensitive to penicillin. Eight consecutive transfers of isolated colonies did not change the properties of the culture, including the production of L forms. The peculiarity of the strain becomes apparent on ascitic agar plates. The colonies develop to uneven size and consist of pleomorphic chains in which some of the cocci swell to large round bodies of 5 μ or more. No L type colonies develop on this medium. A similar pleomorphism develops on horse serum agar if growth is inhibited by penicillin, but here some of the large bodies grow into L type colonies.

The growth of these *L colonies* is markedly influenced by the composition of the medium. It is helped by dextrose, starch, gelatin, and low concentrations of glycine. Animal serum is not necessary, but horse serum makes the growth more abundant. Ascitic fluid and rabbit serum are inhibitory. Anaerobic cultivation is more favorable than aerobic. The most favorable medium found thus far is hard nutrient agar containing 1% gelatin, 2% soluble starch, 0.25% glycine and 10% horse serum. To induce transformation of the cocci into L forms, 200 to 1,000 units of penicillin are deposited at one site on the inoculated plate in a small trough cut into the agar. The plates are incubated anaerobically using Fortner's biological method. Microscopical L type colonies become visible after 24 hours. They require 3 to 5 days for full development. The culture is transferred by a piece of agar cut out with colonies and smeared over a fresh plate. After a few transfers the culture grows abundantly both with and without horse serum. It grows as well without as with high concentrations of penicillin. Growth has never been observed in liquid media. These L type cultures remained stable when transferred every few days on horse serum agar even if penicillin was absent. However, when the cultures were serially transferred on dextrose agar without horse serum and without penicillin, and especially when the plates were kept for several weeks, the cocci reappeared in the cultures. In one case the L forms were cultivated for more than two months in the presence of high concentrations of penicillin (about 1000 units per ml). After a few transfers without penicillin, the cocci reappeared. As in the case of other L forms, it is probable that the cocci were reproduced from the L forms. The cocci could not survive through many transfers on penicillin plates. They were sensitive to penicillin when they were recovered from the L forms. They were never seen in microscopic preparations made from the L cultures and did not grow when the L cultures were transferred to broth. The coccus recovered from the L forms was similar in every respect to the original culture and produced L colonies when exposed to penicillin. This is rare in streptococci and indicates that the recovered strain was identical with the original and was not a contamination. The L type cultures were similar in appearance and morphology to the L forms of other bacteria (1). They were most similar in appearance to the L type cultures of *Salmonella*. The L forms were derived from the cocci in the same way as in the case of other bacteria. The cocci grew to flat, large bodies, sometimes of very large size (10 to 20 μ) and small granules started to grow from these into the medium. Photograph 2 of Fig. 1 shows large bodies developed from the cocci before the growth of granules. The small L colony which developed from a large body shown in photograph 3 of Fig. 1 extends in many directions as strands of small granules into the medium. Photographs 4 and 5 show the appearance of the colonies in stained and unstained preparations. The L forms of streptococcus, like L forms of gram-positive bacilli, are gram-negative. Their morphology is illustrated in photographs 6 through 8 of Fig. 1 and in the electron micrographs in Fig. 2. Electron micrographs made of L type cultures of *Proteus*(2) show, in addition to the elements visible with

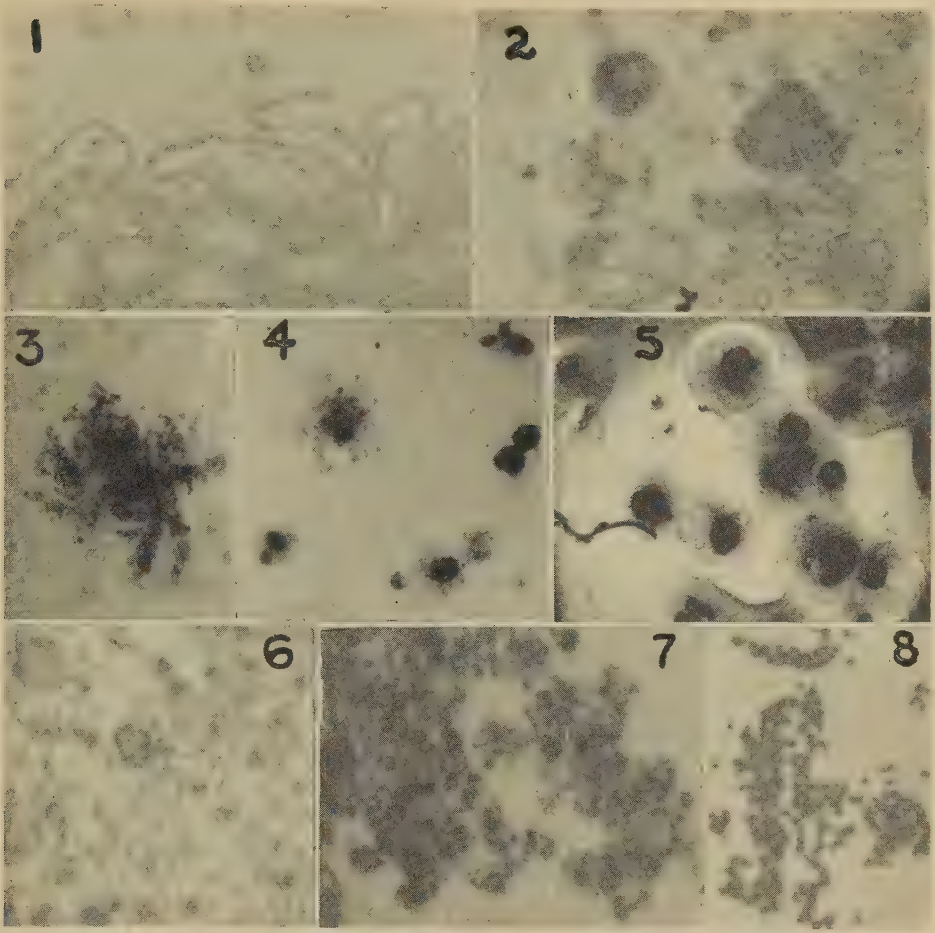


FIG. 1.

1. Edge of a small colony of streptococcus strain 40 on horse serum agar stained with methylene blue on the surface of the medium. × 2000.

2. Cocci and large bodies on an area exposed to penicillin after 24 hours' incubation. The majority of the large bodies are faintly stained and contain small granules. Stained on the surface of the medium. × 2000.

3. A young L-type colony spreading in the medium. Dried stained agar preparation with moderate magnification. × 900.

4. L-type colonies on starch-glycine horse serum plates, inoculated with strain 40 and exposed to penicillin. The colonies are of variable size and several have peripheral growth. Stained as in No. 1 and 2. × 100.

5. L-type colonies as in No. 4, developed to larger size but some remaining very small. Unstained. × 100.

6. Surface growth of L-type colonies suspended in water and stained with crystal violet. × 2000.

7 and 8. Material and staining similar to that in No. 6, but preparations dried. In the area shown in No. 8, the large bodies all disintegrated into small granules. × 2000.

the light microscope, granules 0.3 to 0.1 μ in diameter. Micrographs made from the L culture of streptococcus (Fig. 2) show similar forms. In some parts of the preparation, the size of the smallest granules is between 0.2 and 0.5 μ (Micrograph 3) but at certain places much smaller ones (0.1 to 0.2 μ) are also present. That granules of the size of 0.3 to 0.5 μ may be viable is indicated by the extension of the young colonies into the agar by such forms. The smallest granules (0.1 to 0.2 μ) are produced in the large bodies

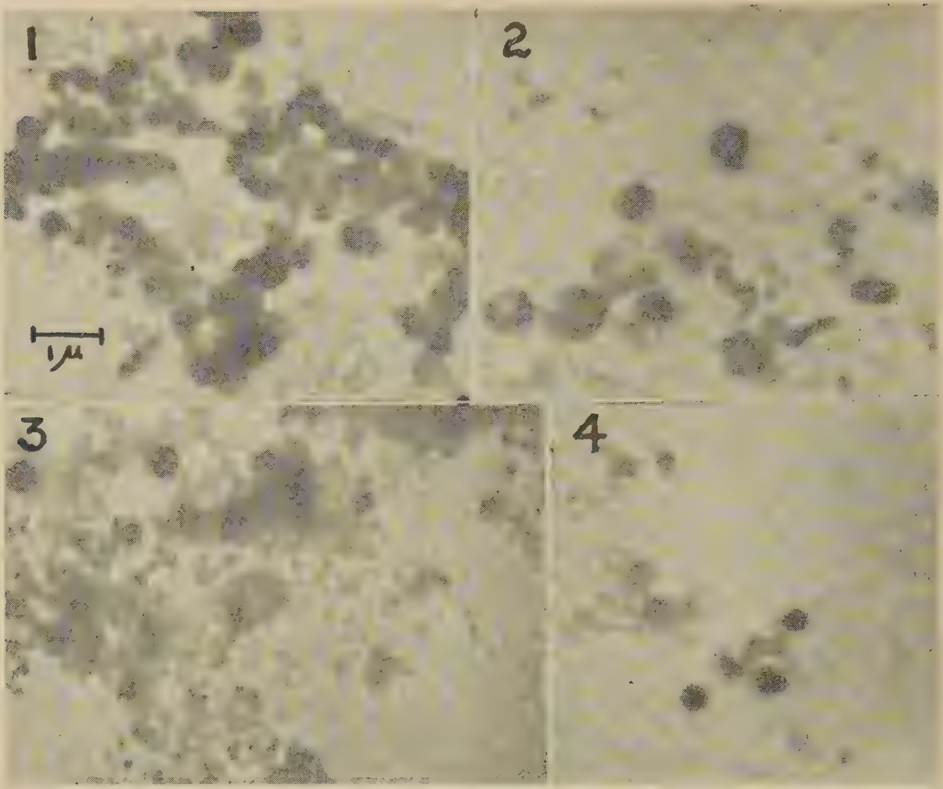


FIG. 2. Electron micrographs made from impressions from agar cultures. × 9000. Most organisms are between 0.5-1.0 μ, but many are considerably smaller. In micrograph 3, the small granules were probably produced by disintegration of larger forms. In micrograph 2, the density is uneven in several of the larger organisms.

and are freed by their disintegration. Nothing definite can be said at present about their nature.

A rabbit antiserum produced with the L strain agglutinated the L strain and the parent streptococcus. It did not agglutinate 3 other alpha-hemolytic streptococci or the L type cultures produced by streptococcus *strain Ch 45*. The L forms were not agglutinated by antisera produced against 2 heterologous coccus strains, or by antisera against oral strains of pleuropneumonia-like organisms. The serological similarity of the L forms and of the coccus is additional evidence for their essential identity.

Streptococcus *strain Ch 45* behaved essentially similarly to *strain 40*. It produced L type colonies under similar conditions, and the cocci reappeared in these in a similar way. However, marked differences were present between the 2 strains. The tendency to produce L forms decreased rapidly in *strain Ch 45* and at the same time the properties of the L cultures changed. The L colonies obtained immediately after isolation of the strains resembled the 3B L type colonies of *Proteus* and *Salmonella*(1). The organisms grew to large bodies not only on the surface but also in the depth of the colonies, and after a few days the cocci reappeared in many of the large bodies. The L colonies isolated somewhat later grew abundantly but reproduced cocci only occasionally in aging cultures. The viability of L type colonies produced by the coccus further decreased, and within a month after isolation the strain lost entirely the tendency to produce L forms and was not made pleomorphic by penicillin.

Many strains of alpha- and beta-hemolytic streptococci were picked from throat and genital cultures and examined for the production of L forms. Thus far only the two strains described produced such forms. However, on plates inoculated from the mouth, colonies similar to the L type colonies of streptococcus often develop, occasionally in great numbers, in the area inhibited by penicillin. It is probable that they are produced by streptococci. Their appearance and size distinguish them from the oral pleuropneumonia-like organisms, and their growth in the absence of animal serum distinguishes them from the L type colonies sometimes produced in the plates by gram-negative bacilli. An L strain obtained from a mouth culture has been carried in our laboratory. It gives good agglutination with an anti-streptococcus rabbit serum, and on one occasion streptococci have been recovered from it. The connection between this streptococcus strain and the L type culture is at present under study. If the above-mentioned colonies develop from streptococci, they indicate that there are usually a few, and occasionally many, streptococci in the mouth which produce L type colonies. There is some evidence that most of these cocci lose the tendency to produce L forms after they start to multiply on artificial medium. If the penicillin is not put on the plates immediately after inoculation but after the bacteria multiply for a few hours, no L type colonies corresponding to those of streptococci will develop. This explains probably the difficulty in isolating streptococcus strains producing L forms. The variability of the tendency of different strains to produce L forms and the diminution of this tendency on cultivation have been observed in many other species producing L forms.

Discussion. The observation of L forms in streptococci adds a new important group of bacteria to those in which L forms have been observed previously. Their occurrence in streptococci is of special interest because of the claims repeatedly made for the production of filterable forms by these cocci. It is impossible to tell at present whether the observation of the L forms will confirm some of these claims.

The concept of L forms was derived from the observation that bacteria sometimes grow with a changed morphology consisting of small granules and large bodies. In the instances described in this paper, the L forms were grown in pure culture, and their identity with the bacteria is supported by good evidence. Recently Klieneberger-Nobel(3) and some other authors following her(4) designated as L forms granules and pleomorphic bacterial forms without offering evidence that these forms reproduce independently from the bacteria. Such use of the term "L form" can create only confusion because certainly not all pleomorphic forms, and especially not all granules visible in bacterial cultures, are analogous to L forms.

Summary. L cultures were isolated from two strains of alpha-hemolytic streptococci obtained from the human mouth. The appearance of these cultures and their morphology are similar to those of other bacteria, and they originated in a similar way from the parent streptococci. When exposed to certain influences, especially to penicillin, the cocci grew to large bodies and, on appropriate media, L type colonies developed from these. The L forms of streptococcus are not sensitive to penicillin and, like the L forms of gram-positive spore-bearing bacilli, do not require animal serum for growth. The cocci reappeared several times in cultures of the L form. Thus far, only two strains of streptococci could be induced to produce L forms. However, colonies similar in appearance and growth requirements to the L type colonies of streptococcus often develop on plates inoculated from the human mouth, suggesting that streptococci which produce L forms are often present in the mouth.

---

1. Dienes, L., and Weinberger, H. J., *Bact. Rev.*, 1951, v15, 245.

2. Dienes, L., *J. Bacteriol.*, 1953, in press.

3. Klieneberger-Nobel, E., *J. Gen. Microbiol.*, 1949, v3, 434.

4. Wittler, R. G., *J. Gen. Microbiol.*, 1951, v5, 1024.

Received May 26, 1953. P.S.E.B.M., 1953, v83.

Cultivation of Yeast in Earle's "L" Strain Mouse Cells *in vitro*.* (20425)

CHARLES C. RANDALL AND DOROTHY J. TURNER.

From the Department of Pathology, Division of Bacteriology, Vanderbilt University School of Medicine, Nashville, Tenn.

In this communication, we shall describe the use of Earle's strain "L" mouse cells(1) as an adjunct to the experimental study of histoplasmosis. This type of cell has been shown by Scherer(2,3) to support the growth of several viruses. Histoplasmosis is an important disease in some areas, particularly in the southern part of the United States, but studies of the experimental disease, in our experience, are handicapped for want of a uniformly susceptible experimental animal. Furthermore, the effect of fungicidal agents in infected animals is difficult to interpret. In an effort to find more suitable animal material in which to study the disease, cultures of Earle's strain "L" cells growing on glass were inoculated with the yeast cell phase of *Histoplasma capsulatum* (H.C.). We have shown previously that H.C. develops intracellularly in tissue cultures of horse and chick tissues(4) but the method is laborious and not as suitable as the one to be described.

Experimental procedure. The animal cells used in this study consisted of Earle's strain "L" cells derived from a single cell(1), grown on glass in Carrel flasks. The medium, changed 3 times weekly, consisted of 1 ml of the following composition: 40% horse serum, 20% chick embryo extract (E_{50}), and 40% Earle's balanced salt solution (BSS). Cultures 8 to 10 days old were scraped with curved-tip pipettes and thoroughly distributed in the liquid phase. Two drops of the resulting cell suspension were placed on a 6 x 22 mm coverglass, allowed to stand for ½ hour at room temperature, and the coverglass was inserted into a sterile rubber-stoppered 16 x 150 mm pyrex test tube containing 1 ml of nutrient fluid. The tube was slanted at an angle sufficient to bathe the surface of the culture and incubated at 37°C. After 2-5 days' incubation, the tissue cells were growing luxuriantly on glass and were judged satisfactory for inoculation of yeast cells of a laboratory strain of H.C. (strain 603 Vanderbilt). The inoculum was prepared by emulsifying two loopsful of a 7-day-old culture in 5 ml of normal saline. The animal cells were inoculated by two different methods. A) The nutrient fluid from one set of tubes was aspirated and 2 drops of the yeast cell preparation were pipetted on the coverglass. After standing for ½ hour at 4°C, the coverglass was rinsed thoroughly with BSS and reinserted into the standard test tube containing nutrient fluid. B) Another set of tubes was inoculated by adding 2 drops of yeast cell suspension directly to the nutrient fluid. All tubes, irrespective of the method of inoculation, were incubated at 37°C and were examined daily for pH and microscopic changes. Fluid change was made 3 times weekly. All preparations were fixed in Zenker-acetic acid at irregular intervals from 2 to 13 days after inoculation and stained by the hematoxylin-eosin-azure method (H.E.A.). Suitable yeast-free controls were stained at the same intervals. It was of interest to determine if infected cells could be subcultured. Accordingly, a group of 5-day-old "L" strain preparations were inoculated with yeast by the first method and the same routine of inspection and change of nutrient fluid was carried out as before, except that prior to each change of nutrient fluid the cultures were thoroughly washed with BSS. Four days after inoculation a number of slides were scraped and the cells pooled and placed on glass in the manner previously described. The subcultures were stained by the H.E.A. method 2-7 days after being initiated.

Results. Intracellular yeasts were observed in the cells growing on glass (Fig. 1), regardless of the method of inoculation. The preparations in direct contact with yeast cells were more heavily infected than those inoculated into the nutrient fluid. It is to be noted that "L" cells containing only a few yeasts did not

---

* This investigation was supported in part by a grant from Eli Lilly and Co.

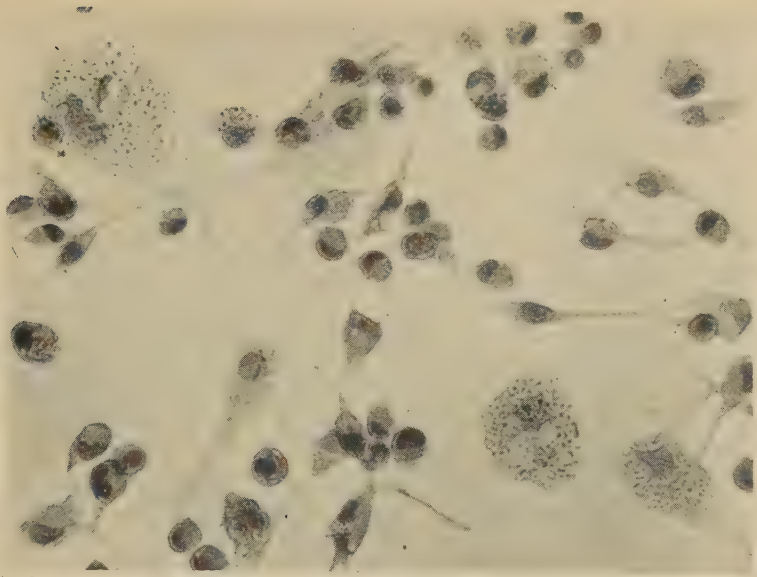


FIG. 1. Six-day-old culture of strain ''L'' mouse cells on glass inoculated with yeast cell phase of *Histoplasma capsulatum* on 2nd day. Stained with H.E.A. Note large cells filled with intracellular yeasts; other cells containing few yeasts are comparable in size to uninfected cells. × 300.

appear significantly larger than the cells of control cultures maintained under identical conditions. However, those containing large numbers of intracellular forms were much increased in size. A comparison of infected cultures of longer incubation periods with those maintained for only a few days showed that the older cultures were usually more heavily infected, though not invariably so. No attempt was made to estimate the relative number of infected cells. Infected cells generally showed little or no degeneration.

The cultures that were grown on glass, subsequently infected with H.C., and subcultured, became readily attached to the glass surfaces. The preparations stained at intervals of 2 to 7 days showed excellent growth of cells, many of which were parasitized.

Discussion. The mouse cells used in the investigation are extremely easy to cultivate and maintain and are easily infected with the yeast cell of *Histoplasma capsulatum.* This system may prove satisfactory in the cultivation of other pathogens, including fungi, and also provide a method of studying the effects of therapeutic agents both on the host cell and on intracellular pathogens.

Summary. It is shown that the yeast cell of *Histoplasma capsulatum* will develop intracellularly in Earle's strain "L" mouse cells cultivated on glass and that infected cells may be subcultured.

---

1. Stanford, K. K., Earle, W. R., and Likely, G. D., *J. Nat. Cancer Inst.*, 1948, v9, 229.

2. Scherer, W. F., *Am. J. Path.*, 1953, v29, 113.

3. ———, *Fed. Proc.*, 1953, v12, 1321.

4. Randall, C. C., and McVickar, D. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 150.

Received May 27, 1953. P.S.E.B.M., 1953, v83.

Further Studies on Effect of Folic Acid Analogs on Growth of *Tetrahymena*. (20426)

VIRGINIA C. DEWEY AND G. W. KIDDER.*

From the Biological Laboratory, Amherst College, Amherst, Mass.

Folic acid analogs in which the hydroxyl group in position 4 is replaced by an amino group are potent inhibitors of the growth, not only of mammals, but also of bacterial and leukemic cells(1-3). However, both bacteria and leukemic cells may develop resistance to such inhibitors(4-8). *Tetrahymena* is naturally resistant to aminopterin and for that reason it was felt that other folic acid analogs capable of inhibiting the growth of *Tetrahymena* might prove useful in inhibition of aminopterin resistant cells.

A number of 3′, 5′ dichloro-substituted folic acid derivatives(9) as well as a number of pteroyl compounds containing an amino acid residue other than that of L-glutamic acid(10) have been tested for their effect on the growth of *Tetrahymena*.

Materials and methods. Tetrahymena pyriformis (geleii) W(11) was used. The media and other conditions of growth have been described previously(12,13).†

Results. Of the 20 folic acid analogs tested, it was found that 2 had the ability of replace folic acid for growth, although the activity was very low. Pteroyl-D-glutamic acid (concentrations from 0.01 to 0.1 γ/ml tested) had an activity of 3.6% and 4-dimethylaminopteroylglutamic acid, 0.43% (concentrations from 0.01 to 1.0 γ/ml tested). The analogs containing various amino acid residues in place of or in addition to glutamic acid (Table I) all showed some activity in sparing folic acid, although none would replace it when tested in concentrations up to at least 0.1 γ/ml. Such of these analogs as contained amino acids required by *Tetrahymena* were also tested for their ability to replace the amino acid. None showed any evidence of activity in this respect at concentrations up to 100 γ/ml. One compound, 3′, 5′-dichlorodiopterin exhibited both sparing and inhibitory effects.

TABLE I. Compounds Having a Sparing Effect on Folic Acid.

Compound	Conc. range tested (γ/ml)
4-dimethylamino-N^{10}-methyl PGA*	.1 –50.
Pteramidomalonic acid	.1 –50.
4-aminopteramidomalonic acid	.1 –50.
Pteroyl-DL-alanine	.1 – 1.0
Pteroyl-DL-seryl-L-glutamic acid	.01– .1
4-aminopteroyl-DL-seryl-L-glutamic acid	.01– .1
4-aminopteroyl-DL-valine	.01– .1
4-aminopteroyl-DL-isoleucine	.01– .1
4-aminopteroyl-DL-serine	.01– .1
3′, 5′-dichlorodiopterin*	.05– .5
Pteroylglycine	.1 –10.

* Inhibitory at higher levels.

The remaining 9 compounds were all competitive antagonists of folic acid (Table II). Only one, 3′, 5′-dichloro-N^{10}-methylpteroylglutamic acid had an inhibitory index small enough to give promise of usefulness.

It has recently been demonstrated(14,15) that aminopterin is grossly contaminated with folic acid. Material obtained as a major peak from the ion-exchange column was found to have no effect on the growth of *Tetrahymena*, either stimulatory or inhibitory. A minor peak showed all the characteristics of folic acid and was active in promoting growth at equivalent concentrations.

Discussion. The evidence now indicates that the variations in the inhibition index obtained with A-methopterin and A-denopterin(16) are probably due to the presence of folic acid or methyl folic acids in the original material rather than to deamination by the organisms as originally supposed. This may also explain the ability of these analogs to substitute for aminopterin in the growth of aminopterin-resistant bacteria. Folic acid

---

* Aided by grants from the Research Corporation and American Cancer Society, recommended by the Committee on Growth.

† We wish to thank Dr. J. M. Smith, Jr. of the Calco Chemical Division, American Cyanamid Co. for generous gifts of the folic acid analogs used.

TABLE II. Compounds Antagonistic to Folic Acid.

Compound	Inhibitory index	Conc. range tested (γ/ml)
N^{10}-methylpteroylglutamic acid*	‡	.1 – 20.
4-aminopteroylglutamic acid	i§	.001– .1
4-dimethylaminopteroylglutamic acid	1600	.001– 1.
3′, 5′-dichloropteroylglutamic acid	500–600	.1 – 5.
3′, 5′-dichloro-4-aminopteroylglutamic acid	600	.1 – 64.
3′, 5′-dichloro-N^{10}-methylpteroylglutamic acid	15– 20	.05 – 2.
4-amino-N^{10}-methylpteroylglutamic acid*	4	.1 – 50.
9-methylpteroylglutamic acid*	500–750	1.0 – 16.
4-amino-9-methylpteroylglutamic acid*	800	.1 –100.
9, 10-dimethylpteroylglutamic acid	200–300	.1 –160.
4-amino-9, 10-dimethylpteroylglutamic acid*	10	.01 –100.
3′, 5′-dichloro-9, 10-dimethylpteroylglutamic acid	125–175	.1 – 8.
3′, 5′-dichloro-4-amino-9, 10-dimethylpteroylglutamic acid	150	.1 –400.
3′, 5′-dichlorodiopterin†	1700	.5 – 80.

* See reference 13.

† 3′, 5′-dichloropteroyl-α-glutamyl-glutamic acid.

‡ Inhibition slight and variable at high levels.

§ i = inert.

contamination is also probably the explanation of the effects of pteroyl-D-glutamic acid and 4-dimethylaminopteroylglutamic acid.

It is interesting to note that the introduction of the 4-amino group into 3′, 5′-dichloropteroylglutamic acid has little effect on the inhibitory index, while its introduction into either of the methylated folic acids greatly increases the inhibitory effect. This increase in inhibition is also obtained by the introduction of the dichloro substitution into the 10-methyl compound. Neither alone gives much inhibition; together they give one of the most inhibitory of the compounds studied.

Of the dihalogenated compounds reported by Cosulich *et al.*(9) the most inhibitory appear to be those with the 4-amino substitution, especially when this is combined with a 9- and/or 10-methyl substitution. However, these results apply to a strain of *Streptococcus faecalis* sensitive to aminopterin. It is possible that cells with a resistance to aminopterin, either natural or acquired, would show a different pattern of response to the halogenated analogs, as does *Tetrahymena*. For this reason we suggest that 3′, 5′-dichloro-N^{10}-methylpteroylglutamic acid might prove to be a better inhibitor of aminopterin-resistant cells than it is of the sensitive cells.

The fact that the amino acid analogs of folic acid cannot be utilized as sources of amino acids indicates that the bond between the *p*-aminobenzoic acid and the amino acid portion of the molecule cannot be hydrolyzed. This would indicate that folic acid cannot be utilized as a source of PABA(17). How these compounds act in sparing folic acid is difficult to explain unless certain apoenzymes exhibit little or no specificity for the amino acid portion of the folic acid molecule.

Summary. 1. The growth of *Tetrahymena pyriformis,* which was produced when aminopterin was added to a folic-acid deficient medium, was due to the presence of folic acid in the aminopterin. 2. A similar contamination may explain the replacement effects of pteroyl-D-glutamic acid and 4-dimethylaminopteroylglutamic acid. 3. Folic acid is spared by a number of folic acid analogs in which the glutamic acid is replaced by other amino acids. 4. These compounds cannot serve as sources of amino acids. 5. Of several 3′, 5′-dichloro-substituted folic acid analogs, the most inhibitory is 3′, 5′-dichloro-N^{10}-methylpteroylglutamic acid. 6. It is suggested that this compound may also be effective in inhibiting aminopterin-resistant cells.

---

1. Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 398.

2. Franklin, A. L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., and Jukes, T. H., *J. Am. Chem. Soc.,* 1949, v71, 3549.

3. Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer,* 1949, v2, 113.

4. Foley, G. E., *Blood,* 1952, v7, 165.

5. Burchenal, J. H., Waring, G. B., and Hutchison, D. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 311.

6. Hutchison, D. J., and Burchenal, J. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 251.

7. Burchenal, J. H., Robinson, E., Johnston, S. F., and Kushida, M. N., *Science,* 1950, v111, 116.

8. Law, L. W., and Boyle, P. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 599.

9. Cosulich, D. B., Seeger, D. R., Fahrenbach, M. J., Roth, B., Mowat, J. H., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.,* 1951, v73, 2554.

10. Wright, W. B., Jr., Cosulich, D. B., Fahrenbach, M. J., Waller, C. W., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.,* 1949, v71, 3014.

11. Corliss, J. O., *Trans. Am. Mic. Soc.,* 1952, v71, 159.

12. Dewey, V. C., Parks, R. E., Jr., and Kidder, G. W., *Arch. Biochem.,* 1950, v29, 281.

13. Kidder, G. W., Dewey, V. C., and Parks, R. E., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 88.

14. Broquist, H. P., personal communication.

15. Heinrich, M. R., Dewey, V. C., and Kidder, G. W., *J. Amer. Chem. Soc.* (in press).

16. Dewey, V. C., Kidder, G. W., and Parks, R. E., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 91.

17. Dewey, V. C., and Kidder, G. W., *J. Gen. Microbiol.* (in press).

Received May 28, 1953. P.S.E.B.M., 1953, v83.

Influence of Dietary Factors and the Adrenal on Hyaluronidase Spreading Reaction in the Rat.* (20427)

EDITH M. BECK AND ELAINE P. RALLI.

From the Department of Medicine, New York University College of Medicine, New York University-Bellevue Medical Center.

The influence of adrenalectomy and adrenal cortical hormones on the spreading reaction of hyaluronidase has been studied in mice and rabbits(1). Conflicting results have been reported in the rat(2,3). The present study is concerned with observations on the intradermal spread of hyaluronidase in intact, cortisone treated intact, in adrenalectomized or sham operated rats, and the influence of nutritional factors on the reaction.

Materials and methods. A total of 185 adult male and female rats, bred in the laboratory, were used in these experiments. Both intact and adrenalectomized rats received a 1% sodium chloride solution as drinking water. No hormonal therapy was given the adrenalectomized rats. One group of intact rats was given 5 mg of cortisone intramuscularly daily for 4 days prior to the skin test. One hour before the skin test was performed another dose of cortisone was given. The rats received one of the following *diets:* 1) a dog chow diet; 2) an experimental diet adequate in all nutritional substances for the rat; 3) the experimental diet supplemented with excessive amounts of calcium pantothenate (43.6 mg per 100 g of diet); 4) the experimental diet supplemented with large amounts of vit. B_{12} (50 μg of B_{12} concentrate/100 g diet); and 5) the experimental diet deficient in pantothenic acid. The composition of the experimental diet (per 100 g diet) was as follows: vitamin free casein 22 g, sucrose 62 g, primex 9 g, salt mixture (ferric citrate 2.3%, calcium phosphate dibasic 44%, potassium citrate 42%, magnesium citrate 11.6%, copper sulfate 0.8%, and potassium iodide 0.01%) 5 g, cod liver oil 2 g, thiamine 0.3 mg, pyridoxine 0.3 mg, riboflavin 0.5 mg, folic acid 0.1 mg, biotin 0.05 mg, calcium pantothenate 1.0 mg, niacine 1.0 mg, choline 30.0 mg, inositol 5 mg, mixed tocopherols 7.5 mg, and synthetic vit. K 5 mg. The animals on the dog chow diets were divided into 4 groups. *One group* was skin tested as a control group, *one* received cortisone intramuscularly for 4 days before the skin test, and the *third* group was bilaterally

---

* This research was aided by a grant from the Josiah Macy Jr. Foundation.

adrenalectomized and skin tested at varying intervals of time postoperatively. A *fourth* group fed dog chow was sham operated and skin tested at similar postoperative intervals.

The animals on the synthetic diets were divided into 5 groups. One group received the pantothenate deficient diet for 30 days; one-half of this group was bilaterally adrenalectomized on the 24th day of the diet. The remaining 4 groups received the control experimental diet for 10 days. At this time one-half of each group was bilaterally adrenalectomized, and all the animals thereafter received one of synthetic diets throughout the remainder of the study. The rats consumed approximately 10-15 g daily of their ration. The *hyaluronidase spreading reaction* was measured 6 to 21 days after adrenalectomy. Food and fluids were withheld 18 hours prior to the injection of hyaluronidase. The test was carried out as follows: a solution of India ink was added to an equal volume of hyaluronidase† in physiological saline so that the concentration of the enzyme was 15 mg/ml. Saline was substituted for the enzyme in the control solution. The animals were lightly anesthetized with ether, the flanks were shaved, and 0.08 ml of the hyaluronidase-India ink solution was injected intradermally into the left flank. An equal volume of the control solution was intradermally injected into the right flank. The injection time was constant at 8 seconds. Six hours later, the animals were sacrificed with illuminating gas and were skinned. The skin was stretched on a flat surface, and 24 hours after the injection the areas of spread were outlined on cellophane and traced onto paper. The area in square centimeters was determined with a planimeter. The results are expressed in square centimeters as the spread of the hyaluronidase-India ink solution minus the spread of the saline-India ink.

Results. The effect of adrenalectomy or the intramuscular injection of 5 mg of cortisone on the spreading reaction in rats fed a dog chow diet is compared with that of control rats in Table I. Control intact rats fed the dog chow diet had a mean area of spread of 8.05 sq cm; adrenalectomized rats 14 days postoperatively had a mean area of spread of 0.47 sq cm; intact rats treated with cortisone had a mean area of spread of 2.29 sq cm. In the rat, both the withdrawal of adrenal hormones and the addition of cortisone appeared to decrease the spreading reaction. Throughout the study no sex difference was found.

TABLE I. Effect of Adrenalectomy or Cortisone Injections on Hyaluronidase Spreading Reaction in the Rat on a Dog Chow Diet.

	No. of rats	Area of spread in cm² (mean ± S.E.)
Intact	23	8.05 ± .81
Adrenalectomized	7	.47 ± .13
Cortisone treated, intact	13	2.29 ± .39

In order to evaluate the effect of the operative procedure, *per se,* 34 rats on the dog chow diet were sham operated and the spreading reaction measured at intervals of 2, 5, 16, and 22 days later. The spreading of the sham operated rats was compared with that of rats on the dog chow diet, adrenalectomized for the same periods of time. At 2 days after operation, the mean area of spread in square centimeters for 5 sham operated rats was 1.90 ± 0.47 compared with 2.05 ± 0.43 for 12 adrenalectomized rats. At 5 days the corresponding values were 2.25 ± 0.44 sq cm for 6 sham operated rats and 1.41 ± 0.22 sq cm for 5 adrenalectomized animals; at 14 days, 1.59 ± 0.14 sq cm for 11 sham operated and 0.47 ± 0.13 for 14 adrenalectomized rats; and at 22 days 3.76 ± 0.65 sq cm for 12 sham operated and 2.16 ± 0.47 sq cm for 5 adrenalectomized animals. All these values are significantly less than the mean area of spread 8.05 ± 0.81 sq cm of intact rats on the dog chow diet. The results indicate that sham operation and adrenalectomy had similar effects on the hyaluronidase spreading reaction in rats.

The effect of various diets upon the spreading reaction in both intact and adrenalectomized rats is shown in Table II. In all groups except those fed a high vitamin B_{12} diet, the adrenalectomized animals had a significant decrease in the areas of spread ($P < 0.05$). Neither a high pantothenic acid diet nor a

---

† Wyeth Co. lot No. W-108-A.

TABLE II. Effect of Diet upon Hyaluronidase Spreading Reaction in Intact and Adrenalectomized Rats.

Diet	Intact			Adrenalectomized			
	No. of rats	Days on diet	Area of spread in cm^2 (mean ± S.E.)	No. of rats	Days on diet	No. days post-op.	Area of spread in cm^2 (mean ± S.E.)
Control experimental	8	30	5.19 ± 1.32	10	30	21	.96[†] ± .19
High pantothenate	7	27	4.21 ± 1.21	11	17	17	1.33[†] ± .39
Pantothenate deficient	11	29	7.35 ± .93	10	30	6	.13[†] ± .16
High vit. B_{12}	8	20	1.72[*] ± .27	8	20	20	1.05 ± .18

[*] Significant difference ($P < .05$) from intact control value.

[†] ” ” ” between intact and adrenalectomized rats on same diet.

pantothenate deficient diet in intact rats significantly altered the area of spread from the control value of 5.19 sq cm. Intact rats fed a high vit. B_{12} diet had a significant decrease ($P < 0.05$) in the area of spread when compared to the control intact rats. The area of spread was reduced from 5.19 sq cm in the control rats to 1.72 sq cm in the animals fed the high vit. B_{12} diet. Adrenalectomized rats fed the high vit. B_{12} diet had a mean area of spread of 1.05 sq cm, which was not significantly different from the intact rats fed the same diet.

Discussion. Adrenalectomy had the opposite effect on the spreading reaction of hyaluronidase in rats than has been reported for mice and rabbits(1). Furthermore, in the rat, sham operation caused as great and as prolonged a decrease in the area of spread as did adrenalectomy. This suggests that the operative procedure is itself an important factor in the spreading reaction in this species, and that the effect of adrenalectomy may be non-specific. The injection of cortisone decreased the spread of hyaluronidase and in this respect the rat behaved as do other species(1). The fact that adrenalectomy and injection of the adrenocortical hormone both reduced the area of spread also supports the suggestion that the effect of adrenalectomy on the spreading reaction was not specific.

The spreading reaction was also decreased in intact rats by the administration of excessive amounts of vit. B_{12} in the diet. The explanation of this reaction is not clear. We did observe that the skin of the rats on vit. B_{12} appeared to be thicker than that of the rats on other diets. However, no measurements of skin thickness were made. Hayes *et al.*(4) have noted that prolonged local therapy of the skin with adrenal cortical extract causes thinning of the dermis and epidermis of the rat, and under these conditions they noted an increased spread of hyaluronidase.

In the rats on the pantothenate acid deficient diet, there was a moderate increase in the area of spread following the injection of hyaluronidase. Pantothenic acid deficiency causes atrophy of the hair follicles and bulbs in the rat(5) and obvious thinning of the skin. One might, therefore, anticipate that under these circumstances the intradermal injection of the hyaluronidase solution would result in a greater spread than in animals on a normal diet. The rats in this experiment were maintained on the deficient diet for only 29 days, and possibly prolonging the period of the deficiency would have resulted in a more pronounced effect on the spreading reactions. Pantothenate deficiency also affects the integrity of adrenal cortical cells(6,7), but from the present data there is no way of evaluating the effect of the adrenal damage on the spreading reaction. The diet containing excessive amounts of pantothenate had no influence on the area of spread of hyaluronidase.

The observations reported indicate that in the rat the effect of adrenalectomy on the hyaluronidase spreading reaction is non-specific. Cortisone in large doses does inhibit the intradermal spread of hyaluronidase. The effect of the dietary situations on the spreading reaction is most probably due to the local influences of nutritional fractions on the skin apparatus of the rat.

Summary. 1. The hyaluronidase spreading reaction was tested in intact, cortisone injected and adrenalectomized rats and the effect of various dietary situations was studied. In intact rats, a diet containing excessive amounts of vit. B_{12} decreased the area of spread significantly. At present, there is no obvious explanation for this observation. 2. Both the administration of cortisone and adrenalectomy decreased the area of spread in rats. Sham operation also decreased the area of spread of hyaluronidase. It is suggested that the effect of adrenalectomy is primarily a non-specific effect due to the operative procedure itself.

---

1. (a) Opsahl, J. C., *Yale J. Biol. and Med.*, 1949, v21, 255; (b) *Ibid*, 1949, v21, 487.

2. Winter, C. A., and Flataker, L., *Fed. Proc.*, 1950, v9, 137.

3. Hayes, M. A., and Baker, B. L., *Endocrinology*, 1951, v49, 379.

4. Hayes, M. A., Reed, T. G., and Baker, B. L., Proc. Soc. Exp. Biol. and Med., 1950, v75, 361.

5. Ralli, E. P., and Graef, I., *Endocrinology*, 1945, v37, 252.

6. Morgan, A. F., and Simms, H. D., *Science*, 1939, v89, 565.

7. Daft, F. S., and Sebrell, W. H., *Public Health Reports*, 1939, v54, 2247.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Free Wandering Cells and Cortisone. (20428)

George H. Paff and Robert Stewart.

From the Department of Anatomy, University of Miami, School of Medicine, Coral Gables, and the Department of Biology, Swarthmore College, Swarthmore.

After Bloom(1) reported that cortisone could produce a rapid regression of mast cell tumors in dogs, we began a study of the effects of cortisone on the mast cell in tissue culture. For reasons which are not clear to us, the rapidly destructive effects on the mast cell obtained by Bloom *in vivo* were only mildly demonstrable *in vitro*. Mast cell activity was inhibited, it is true, but this was only a facet of a broader observation, *i.e.*, cortisone can effectively block the nomadic proclivities of free wandering cells. This agrees with previous observations on leucocytes(2,3) but the fact seems to have been overlooked that not only white blood cells but free wandering cells in general are more sensitive to the hormone than are fibroblasts. All free wandering cell activity can be brought to a stop in cultures in which fibroblastic activity remains relatively undisturbed.

Materials and methods. Coverslips (43 x 50 mm) containing crystals of cortisone (free-alcohol) in amounts ranging from 0.0005 to 0.025 mg were prepared by permitting the hormone to precipitate by evaporation from measured volumes of an alcohol-cortisone solution. On these coverslips fragments of 9-10 day old embryonic chick skin were placed in equal parts of blood plasma and embryonic extract before the medium clotted. Controls contained either no hormone, or cholesterol in amounts similar to the amount of hormone used in the experimental cultures. More than 200 pairs of embryonic skin fragments were grown and observations were taken each day over a period of 4 days. We also studied about 200 pairs of spleen fragments obtained from 16-19 day old chick embryos. Here the technic was varied in that, with both the experimental and control cultures, the medium was permitted to clot before a spleen fragment was placed meticulously, with no lateral motion, upon the surface of the medium. This procedure was followed in order to minimize the spread of cells by mechanical means. The spleen fragments were taken from 16-19 day old embryos because, at the age chosen for the source of skin material, namely, 9-10 days, the spleen is both undesirably small and soft.

Results. The method of introducing the hormone and the concentrations chosen permitted a graded study of cellular reactions ranging from those which showed no apparent response to the hormone to those in which all activity of all the cells was completely inhibited. The first and most constant effect observed in the skin fragments subjected to cortisone was the failure of the free wandering cells to migrate from the parent fragment into the surrounding medium. Significantly, this retardation of migratory activity of the free wandering cells occurred in many cultures in which there was no apparent interference with the growth and movement of fibroblasts. In brief, the free wandering cell is distinctly more sensitive to cortisone than is the fibroblast. Following the failure of free wandering cells to migrate, the first apparent reaction of the fibroblast to the hormone is the formation of cytoplasmic vacuoles. A few cells show distinct vacuoles at the end of one day of cultivation. By the end of 2 days this number has increased to the extent that, in some cultures, it is difficult to find cells free of vacuoles. Finally, the most drastic effect of the hormone is expressed by the complete failure of fibroblasts to leave the parent fragment.

The reaction of spleen fragments to cortisone completely confirms the above observations on skin. Spleen cultures exposed to the hormone showed, at the end of 24 hours, either a marked or a complete suppression of the migratory activity of free wandering cells. This contrasted strikingly with the wide halo of newly migrated hordes of wandering cells seen in the controls. Occasionally it was possible to identify ray-like masses of spindle-shaped fibroblasts extending into the medium from spleen fragments treated with hormone. It is apparent that these rays were unmasked by the wandering cell inhibitory action of cortisone.

Discussion. The term free wandering cell as used in this report is a blanket term for any and all cells which leave the parent fragment to travel singly through the meshes of the medium, over the surface of the medium and between the groups of fibroblasts. It includes the various types of white blood corpuscles, the so-called lymphoid wandering cells, macrophages, and also the mast cell.

Regarding the experimental results, it does not necessarily follow that the unfavorable reaction which the free wandering cell exhibits toward cortisone *in vitro*, will occur *in vivo*. The amount of cortisone which reached the cells in the cultures is not known. All of the cortisone deposited upon a coverslip does not dissolve and the rate of diffusion is very slow.

Conclusion. In tissue cultures of embryonic skin it can be demonstrated that, of the cell types present, the free wandering cells are the most sensitive to the hormone. All wandering cell movement can be brought to a stop in cultures in which fibroblastic activity remains undisturbed. This observation is supported by the behavior of cortisone-treated spleen fragments.

---

1. Bloom, F., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 651.

2. Holden, M., Seegal, B., and Ryby, I., *Am. J. Path.*, 1951, v27, 748.

3. Barber, M., and Delaunay, A., *J. Path. and Bact.*, 1951, v63, 549.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Effect of Additions to ACD Blood on Erythrocyte Preservation.* (20429)

OTTO SCHALES.

From the Chemical Research Laboratory, Alton Ochsner Medical Foundation and Department of Biochemistry, Tulane University School of Medicine, New Orleans, La.

Human blood, when collected routinely in ACD solution and stored at 4°C is considered suitable for transfusion purposes only during the first 3 weeks after collection. There occurs during storage a gradual change in the red cells so that they lose the ability to survive after entering the circulatory system of the recipient. This process of erythrocyte deterioration is manifested *in vitro* by a progressive increase in osmotic fragility on exposure of red cells to hypotonic sodium chloride solutions. The decisive chemical events responsible for the limited *in vitro* "survival time" of red cells are not known. Enzymatic processes might be at work changing the red cell envelope, either directly through attacks on the lipids and proteins forming the membrane, or, indirectly, through the formation of products injurious to the membrane.

This report describes the effect of a variety of enzyme inhibitors, enzyme activators, and related compounds on the rate of the progressive development of osmotic fragility of human erythrocytes during storage.

Methods. ACD blood collected by the Red Cross was used in this work. In each bottle, as supplied, was a mixture of 480 ml blood and 120 ml ACD solution (NIH solution B, each 100 ml containing 1.47 g dextrose, U.S.P., 1.32 g sodium citrate, U.S.P., and 0.48 g citric acid, U.S.P.). The compounds to be tested for their effect on the development of osmotic fragility were prepared in aqueous solutions, adjusted to pH 6-7, made isotonic by addition of the calculated amount of NaCl, and sterilized by passage through a Seitz filter. If this sterilization method was accompanied by undesirable reactions (for example oxidation of the phenothiazine derivative mentioned below to a blue product) filtration through a Morton Filter Apparatus with ultrafine fritted glass disc (Corning No. 33990) was used instead. ACD blood in 50 ml portions was distributed into rubber stoppered sterile bottles, which contained small volumes (up to 5 ml) of the solutions to be investigated. The mixtures, together with a few control bottles containing samples of the ACD blood from the same donor without further additions (unmodified ACD blood) were stored at 4°C. After various periods of storage time, test samples as well as the corresponding controls were examined for chemical changes (N.P.N., glucose, inorganic phosphate, plasma potassium), for changes in red cell volume (hematocrit), and for osmotic fragility. The tests for *osmotic fragility* were carried out as follows. Three 0.1 ml samples of the blood to be tested were transferred to three test tubes containing 20 ml of, respectively, distilled water (tube 1), 0.9% NaCl solution (tube 2), and 0.6% NaCl solution (tube 3). After gentle mixing, the cell debris as well as the cells remaining intact in the NaCl solutions (tubes 2 and 3) were centrifuged down and the supernatant solutions were transferred to colorimeter tubes. Absorbancy measurements were carried out using a Lumetron Photoelectric Colorimeter, Model 402-E, Filter M-550, after addition of a drop (about 0.05 ml) of 2 *N* NH_4OH to the contents (about 10 ml) of each tube. Identical results were obtained, whether the NaCl-blood mixtures were centrifuged immediately when prepared or whether they were left standing for 30 min before centrifugation. The absorbancy of the completely hemolyzed sample (tube 1) was a measure of the total hemoglobin content. The percentage of this total hemoglobin released into isotonic (0.9%) NaCl solution, calculated from the absorbancy of the content of tube 2, represented the percentage of hemolysis which took place during storage. The percentage of the total hemoglobin found additionally in tube 3, containing 0.6% NaCl solution, gave an indication of osmotic fragility

---

* This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service.

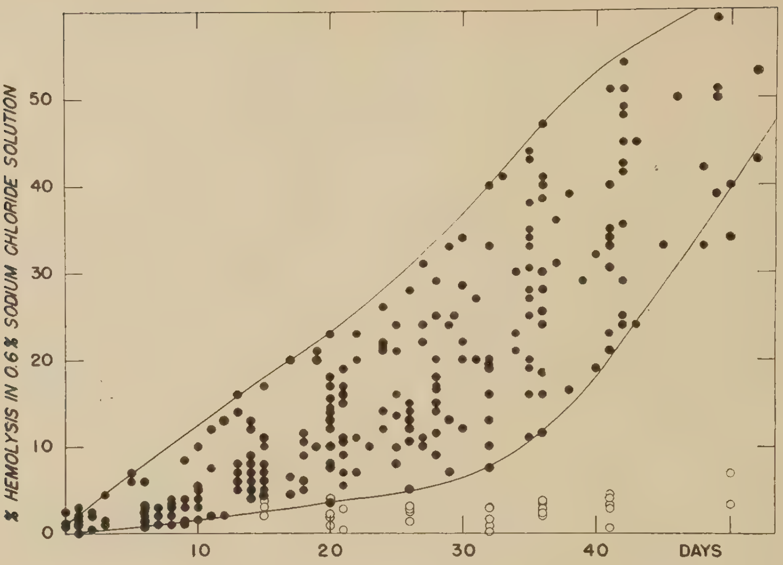


FIG. 1. Increase in osmotic fragility of human erythrocytes during storage of ACD blood. Data obtained with ACD blood from 55 different donors at various intervals are represented by solid black dots. Seven of these specimens were also stored with addition of Phenergan (0.4 mM/l). Results obtained with these modified samples are shown as circles near the abscissa.

and was used as an estimate of *"in vitro survival"* of red cells. This *in vitro* procedure follows a suggestion of and is based on findings by Gibson and coworkers(1) who had reported that the results obtained with this method showed a fair correlation with the post-transfusion survival measured directly by the Ashby technic.

Results of chemical analyses. Neither unmodified ACD blood nor blood samples containing those of the various additions to be discussed later, which retarded the increase in osmotic fragility, showed any significant changes in non protein nitrogen during storage periods of 42 days. This indicated that proteolytic processes leading to *small* breakdown products (soluble in trichloracetic acid) were not taking place. The glucose content of ACD blood and of the modified samples, with the exception of those containing NaF, decreased during storage. A specimen of ACD blood containing, for example, 366 mg glucose per 100 ml just after collection, showed a glucose content of 303 mg after 21 days, and of 269 mg per 100 ml after 42 days of storage. The *plasma potassium* content of ACD blood, of the samples with less pronounced osmotic fragility, and of those containing NaF, increased at about the same rate during storage, for example from 5 meq potassium per liter plasma at the time of collection to 17 meq after 21 days, and 30 meq/liter after 42 days. The concentration of inorganic phosphate in whole ACD blood and plasma increased, for example from 3.2 mg per 100 ml blood on the first day to 13 mg after 21 days, and 15.8 mg per 100 ml of blood after 42 days. The various additions to ACD blood, with the exception of NaF, did not alter significantly the rate of increase of inorganic phosphate.

Changes in hematocrit and osmotic fragility of ACD blood. The hematocrit of ACD blood increased regularly during storage, for example from 44 to 48 in 42 days. There was little spontaneous hemolysis of ACD blood during the early part of the storage period, as measured by the amount of hemoglobin released into 0.9% NaCl solution. Hemolysis after 42 days ranged from 1-3%, but reached 10-13% after 92 days of storage. The osmotic fragility of ACD blood varied with different specimens. Blood from 55 different donors showed on exposure to 0.6% NaCl solution after 21 days storage 5 to 23% (median

TABLE I. Additions to ACD Blood Accelerating the Progressive Increase in Osmotic Fragility during Storage.

Addition	Concentration, mM/l blood	Storage time, days	Hemolysis factor*
L-Arginine	13.6	32	1.28
L-Histidine	.40	24	4.1
Ascorbic acid	1.12	21	1.51
	3.31	21	2.07
Nicotinic acid	.16	29	1.96
Nicotinamide	9.0	57	1.09
Calcium pantothenate	.006	41	1.14
Acetylcholine chloride	1.4	43	1.19
	4.1	43	1.40
p-Amino salicylic acid	.41	39	1.25
	3.27	36	1.34
Benzoic acid	8.33	26	1.62
m-Hydroxy benzoic acid	8.50	34	1.46
p-Hydroxy benzoic acid	8.50	35	2.06
Pyruvic acid	3.0	28	1.18
	11.4	28	1.67
N-Ethyl maleiimide	.73	23	1.83
Methylene blue	.05	41	1.41
	.24	41	1.50
3.7-Diamino-5-methyl-acridinium chloride	.20	30	1.50
α-Hydroxy phenazine	.46	23	1.84
Copper sulfate	.16	10	6.20
	.73	10	10.50
Trypsin (1:300, NBCo.)	10.0†	42	1.32

* Hemolysis factor calculated by dividing percentage hemolysis (in 0.6% NaCl) of test sample by that of control blood.

† mg/liter blood.

13%), and after 42 days 20 to 58% hemolysis. A graphic presentation of such measurements is shown in Fig. 1.

Compounds without effect on osmotic fragility. In blank experiments it was ascertained that the addition of 0.1 volume of isotonic (0.9%) NaCl solution to ACD blood before storage had no effect on the rate of increase of osmotic fragility observed with the corresponding unmodified ACD blood. The following substances were also found to have no influence on the progressive increase in osmotic fragility of red cells during storage (in parentheses are concentrations in millimole per liter blood): ethylenediamine tetraacetic acid (2.0; 4.0; 9.0), iminotriacetic acid (9.0), orthodiaminocyclohexane tetraacetic acid (9.0), nicotinamide (3.0; 6.0), riboflavin (0.001; 0.005), p-amino benzoic acid (0.63; 3.05), creatine (1.2; 3.4), leucine amide (1.0; 4.1), D,L-malic acid (0.4; 9.2), tartaric acid (0.04; 1.96), urea (1.2; 5.8), thiourea (0.12; 0.59), guanidine (0.60), semicarbazide (1.58), sodium thiosulfate (0.40), sodium diethyl dithiocarbamate (0.60; 2.95; 13.7), uric acid (0.02; 0.54), thioglycolic acid (0.78; 3.84), cobaltous acetate (0.28; 1.10), nickel chloride (0.41; 0.81), zinc sulfate (0.20; 0.77), and N,N-dimethyl-N′-(2-pyrimidyl)-ethylenediamine hydrochloride (0.39). Likewise without effect were pepsin (1:10,000; Pfanstiehl, 10 mg/l blood) and cryst. trypsin inhibitor from soybeans (Armour Laboratories; 20 mg/l blood).

Compounds which accelerated the increase in osmotic fragility. Addition to ACD blood of a number of compounds decreased the "*in vitro* survival time" of erythrocytes to a varying degree, as shown in Table I. Results obtained with NaF are given separately in Table II. The presence of NaF during storage did not change the rate of diffusion of potassium into the plasma from that observed with control blood (corrections were made for the potassium released by spontaneous hemolysis during storage).

Compounds which retarded the increase in osmotic fragility. a) *Sodium ferrocyanide* (5.45 mM/l blood). After 21 days storage, the control showed 4.6%, the test sample 2.9% hemolysis in 0.6% NaCl solution. After 50 days the corresponding figures were 28.4% (control) and 14.0% (test sample). b) *Nickel chloride* (1.91 mM/l blood). The effect was very slight. After 29 days, the control showed 29.0%, the test sample 22.7% hemolysis in 0.6% NaCl solution. After 35 days, the corresponding figures were 46.6 and 38.8%, respectively. Lower concentrations of nickel chloride had no effect, as mentioned before. c) *Salicylic acid* (13.63 mM/l blood) showed good protective action for the first 20 days, but this effect all but disappeared when the storage period was extended (Table III). d) *10-(2-Dimethylaminopropyl)phenothiazine.* The addition of optimal amounts of this substance (0.4 mM/l) retarded the development of osmotic fragility to a considerable degree. After 21 days there was observed 0.4 to 4% (controls 5 to 23%), after 42 days

TABLE II. Effect of Sodium Fluoride on ACD Blood during Storage.

Blood No.	NaF, mM/l	Storage, days	% hemolysis in 0.9% NaCl		% hemolysis in 0.6% NaCl		Inorganic phosphate, mg/100 ml blood	
			Control	Test	Control	Test	Control	Test
2341	13.9	2	0	.8	.4	18.7	3.77	3.72
		15	.2	2.4	4.8	62.6	13.54	6.05
		29	.5	8.8	13.4	71.8	16.72	7.57
		35	2.1	12.7	19.3	78.7	18.05	8.66
2342	13.9	2	0	.9	1.9	14.8	3.26	2.86
		15	.6	4.0	3.0	65.5	11.28	4.32
		29	1.0	8.1	7.1	70.5	13.19	5.21
		41	2.1	14.1	21.2	87.5	15.27	5.92
2355	1.5	2	0	.5	0	16.5	3.24	3.03
		16	1.3	5.5	6.1	74.9	12.55	5.06
		30	1.0	11.0	21.7	89.4	15.27	7.79
		49	5.5	18.5	49.7	94.4	17.59	11.44

The following changes in blood glucose were observed during storage periods: Blood 2341, control, glucose decreased from 344 to 250, test sample no change with 354 and 350 mg/100 ml, respectively; Blood 2342, control, glucose decreased from 360 to 286, test sample constant at 368 mg/100 ml; Blood 2355, control, glucose decreased from 332 to 216, test sample no change with 337 and 336 mg/100 ml, respectively. Mixtures of NaF (1.5 mM/l) with cobaltous acetate (2 mM/l), or manganese sulfate (2 mM/l) gave essentially the same results as NaF alone.

TABLE III. Effect of Salicylic Acid (13.63 mM/l Blood) on ACD Blood during Storage.

Blood No.	Storage, days	% hemolysis in 0.6% NaCl	
		Control	Test
2379	10	3.2	1.2
	20	13.6	4.9
	42	50.8	40.3
2382	10	3.7	2.9
	20	22.8	11.3
	42	47.6	45.5
2385	10	5.9	3.1
	20	21.3	11.0
	42	57.5	46.7

0.6 to 4.5% (controls 20 to 36%), and after 92 days 26 to 39% (controls 83 to 85%) hemolysis on exposure to 0.6% NaCl solution. A few examples of the experimental findings are listed in Table IV (see also Fig. 1). The presence of this phenothiazine derivative, which is manufactured under the trade name of Phenergan, did not modify glucose utilization, plasma potassium increase, or inorganic phosphate increase, but it inhibited by 20% plasma lipase activity, determined by the method of Goldstein *et al.*(2), as modified by Alper(3). The increases in hematocrit were less, usually one-half of those observed with ACD alone.

It is important, for reasons to be discussed later, that the addition of Phenergan to ACD blood which had been stored for various periods of time did not modify or reverse the osmotic fragility existing at the time of addition, but showed a marked protective action against further deterioration. This is illustrated by the data shown in Table V.

The methosulfate of Phenergan was also tested but proved to be considerably less effective in retarding the increase in osmotic fragility during storage than the parent compound. The control bottles from 3 different ACD bloods showed, for example, after 25 days 13 to 21%, and after 60 days 47 to 69% hemolysis in 0.6% NaCl solution. Phenergan methosulfate (0.4 mM/l blood) reduced the hemolysis figures at 25 days insignificantly to 11 to 20% and only slightly after 60 days to 41 to 53%. The corresponding values for Phenergan by contrast were 4.6 to 6.8% (25 days) and 12 to 19% (60 days).

Discussion. Metal ions, compounds reacting with sulfhydryl and disulfide groups and certain complex forming substances did not protect red cells from the gradual increase in osmotic fragility. The results with NaF add further weight to the belief that a functioning glycolytic system is essential for *in vitro* preservation of red cells.

A few substances were found to delay the progressive increase in osmotic fragility of

TABLE IV. Protective Effect of 10-(2-Dimethylamino-propyl) Phenothiazine on ACD Blood during Storage.

Blood No.	Storage, days	% hemolysis in 0.6% NaCl Control	Test
606	6	2.4	.8
	20	7.4	1.8
	41	33.1	2.9
2092	11	2.1	2.1
	17	4.5	2.5
	26	9.9	3.2
	32	12.8	3.5
	45	33.0	6.5
	52	43.2	12.8
	59	55.2	19.5
899	21	22.8	2.8
	50	62.9	7.1
	92	84.7	25.6

TABLE V. Effect of Phenergan on Aged ACD Blood.

Age of blood, days	Days stored with Phenergan	% hemolysis in 0.6% NaCl Control	Test
Blood No. 1014, stored 14 days before addition of Phenergan			
15	1	13.2	12.6
24	10	25.8	13.6
34	20	41.0	20.2
Blood No. 2487, stored 37 days before addition of Phenergan			
38	1	54.4	52.8
59	22	80.8	57.3

erythrocytes during storage. The most effective agent among the compounds studied so far was Phenergan hydrochloride. The mechanism of its "protective" action is open to speculation. This substance is known to be an antihistamine, but another antihistamine drug (dimethyl-pyrimidyl-ethylenediamine hydrochloride or Neohetramine) had no stabilizing influence on red cells.

It has been reported that Phenergan preserved tissues and inhibited trypsin and papain(4) and the antiproteolytic activity of this material was our reason for testing its influence on the development of osmotic fragility, especially since Halpern *et al.*(5) found that it retarded morphological changes in stored blood. However, other compounds, as for example ethylenediamine tetraacetic acid (EDTA), which have been shown in a recent report from this laboratory(6) to inhibit certain proteolytic processes, did not duplicate the effect of Phenergan on stored erythrocytes. This is particularly surprising since EDTA, which was used by Dyckerhoff *et al.*(7) as an anticoagulant by virtue of its reaction with calcium, was reported, as had been Phenergan, to keep blood morphologically intact longer than sodium citrate or the oxalates(8).

A third property of Phenergan, found during the course of this study, was its inhibitory effect on plasma lipase activity. While only 20% inhibition was observed when this substance was present in a concentration of 0.4 mM/l blood and when tributyrin was used as substrate, one has to keep in mind that plasma lipase activity is apparently not due to a single enzyme but to a mixture of enzymes. It is conceivable, therefore, that a 20% inhibition of tributyrin hydrolysis might signify a complete inhibition of that part of plasma lipase activity which may be directed against lipids in the red cell membrane. Quinine (2.7 mM/l blood) inhibited plasma lipase activity 66% but caused no significantly increased "*in vitro* survival" of red cells, though these experiments, not reported here in detail, gave somewhat uncertain hemolysis data as a result of blood pigment changes. Tests with p-amino salicylic acid were carried out as this substance was claimed to inhibit plasma lipase quantitatively when present in a concentration of 170 mg/l(9). Experiments in this laboratory showed no inhibition of tributyrin hydrolysis even with p-amino salicylic acid levels of 918 mg/l plasma (6.0 mM/l) and the compound did not interfere with the normal rate of increase of osmotic fragility of red cells during storage. Inability to reproduce the Phenergan effect by using other antihistamines, other antiproteolytic agents, or other lipase inhibitors, makes it impossible at this time to explain the mechanism through which this phenothiazine derivative exerts its protective action.

The data shown in Table V are of particular significance and require additional comment for the following reason. Chaplin *et al.*(10) investigated the effect of a related phenothiazine derivative (R.P. 3300) on red cell preservation, and found that their compound interfered with the hemolysis of fresh red cells by hypotonic NaCl solution. They observed strikingly less hemolysis when small amounts of R.P. 3300 were added to the hypotonic salt solutions. This property of R.P. 3300 made it appear (when blood was stored in its presence for longer periods of time) as if this compound had a protective action, whereas, according to Chaplin *et al.*, it only interfered with the determination of "*in vitro* survival". Transfusion experiments showed that R.P. 3300 did not increase posttransfusion survival.

The behavior of Phenergan in hemolysis experiments was quite different, as can be seen in Table V. In contrast to R.P. 3300, this compound, which was present in the hemolysis tests in a concentration of 1:1.6 million, did not (even after 24 hours contact) decrease the degree of hemolysis from that measured just prior to its addition. Once Phenergan was present, however, it retarded the *further* increase in osmotic fragility observed on continued storage of the control blood.

Experiments are in progress to determine whether Phenergan prolongs the useful shelf life of transfusion blood. It is also planned to study the effect of Phenergan on blood preserved with ethylenediamine tetraacetic acid.

Summary. 1. Based on the working hypothesis that the deterioration of erythrocytes during storage may be due to enzymatic processes affecting the cell membrane, experiments were carried out in which a variety of compounds known to modify enzymatic reactions were added to ACD blood. The effect of these additions on the rate of development of osmotic fragility (hemolysis in 0.6% NaCl solution) was determined. 2. The "*in vitro* survival time" (resistance toward 0.6% NaCl solution) of red cells was considerably shortened by a number of compounds, for example by sodium fluoride, which also prevented the utilization of glucose, delayed the rate of increase of inorganic phosphate during storage, but had no effect on the gradual increase of plasma potassium concentrations. 3. The most promising results in retarding the development of osmotic fragility were obtained with 10-(2-dimethylamino-propyl)phenothiazine. ACD blood from 55 donors showed on exposure to 0.6% NaCl solution after 21 days storage 5 to 23%, and after 42 days 20 to 58% hemolysis. The addition of the phenothiazine derivative (0.4 mM/l blood) retarded the development of osmotic fragility considerably, so that after 21 days there was observed 0.4 to 4%, after 42 days 0.6 to 4.5%, and after 90 days 26 to 39% hemolysis when the blood samples were added to 200 volumes of 0.6% NaCl solution.

The author wishes to thank the staff of the New Orleans Regional Blood Center of the American National Red Cross for their cooperation, and Dr. Joseph Seifter of the Wyeth Institute of Applied Biochemistry for a generous supply of Phenergan.

---

1. *cf.* Sack, T., Gibson, J. G., 2nd, and Buckley, E. S., *Surg., Gynecol., and Obstetr.*, 1952, v95, 113.

2. Goldstein, N. P., Epstein, J. H., and Roe, J. H., *J. Lab. Clin. Med.*, 1948, v33, 1047.

3. Alper, C., *The Clinical Chemist*, 1952, v4, 14.

4. Maral, R., Carraz, M., and Blandin, A., *C. r. Soc. Biol.*, 1950, v144, 136.

5. Halpern, B. N., Dreyfus, B., and Bourdon, G., *Presse Medicale*, 1950, v58, 1151.

6. Schales, O., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 75.

7. Dyckerhoff, H., Marx, R., and Ludwig, B., *Z. f. d. ges. exp. Med.*, 1941, v110, 412.

8. Proescher, F., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 619.

9. Perenyi, L., and Janossy, L., *Kiserletes Orvostudomany*, 1950, v2, 366; from *Chem. Abstr.*, 1951, v45, 8645.

10. Chaplin, H., Jr., Crawford, H., Cutbush, M., and Mollison, P. L., *J. Clin. Path.*, 1952, v5, 91.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Effect of Terramycin on Fecal Microflora of Rats. II. Therapeutic Level of Terramycin.* (20430)

JOSEPH V. SCALETTI, WALTER L. KULP, AND ROBERT C. CLEVERDON.
(Introduced by Edgar Zwilling)
From the Department of Bacteriology, University of Connecticut, Storrs, Conn.

A previous report(1) of the effect of dietary terramycin (100 mg per kg), in 3 rations, indicated statistically significant increases in *Proteus spp.*, regardless of count of *Escherichia coli.* This observation is in disagreement with Sieburth *et al.*(2,3) who reported the simultaneous decrease in *E. coli* and increase in *Proteus.*

In the expectation of obtaining exaggerated effects of terramycin, yielding counts more susceptible of statistical analysis and to verify the previous findings, the experimental procedure previously described(1) was repeated, except that 666 mg terramycin per kg ration was administered, total counts of lactobacilli were made using Rogosa's medium(4), and the experimental period was 5 weeks. Essentially, the procedure consisted of making weekly bacterial counts, using appropriate media and conditions, of feces of groups of animals receiving 3 diets with and without terramycin. The amount of terramycin fed the animals approximated the dosage administered in human therapy. The diets differed in concentrations of protein and carbohydrate. The counts and changes in counts obtained were subjected to a standard analysis of variance(5). Table I includes the mean values of the logarithms of the real numbers, and the degree of variability of the following experimental groupings: (a) among rations, (b) between composited treated and untreated animals, and (c) between replicates. Table II shows the two values making up each subgroup (mean logs of all enumerations) and the subgroup means. The least significant difference (L.D.S.) at the 5% level of probability between subgroup means is also included in Table II.

Results and discussions. The groups of microorganisms studied will be discussed in order of their appearance in Tables I and II.

Total anaerobic count. The addition of terramycin to all rations resulted in a marked decrease in the viable anaerobes ($P<0.001$). This reduction was independent of ration (absence of significant interaction between ration and terramycin). Ration, *per se,* however, exerted an effect on the anaerobic count, but at low probability ($P<0.10$), highest counts being obtained with the high carbohydrate ration.

Total aerobic count. The addition of terramycin to all 3 rations resulted in a marked reduction of aerobic count ($P<0.05$). There was no interaction apparent between diet and terramycin, and no effect of diet *per se.*

Proteus spp. The number of *Proteus spp.* was markedly increased by the addition of terramycin to any of the 3 rations ($P<0.001$). The increase was not influenced by population of *E. coli,* an observation in disagreement with Sieburth and his associates(2,3). Ration *per se* exerted no effect on the *Proteus* count nor was there an apparent interaction between ration and terramycin.

Fecal streptococci. Terramycin *per se* resulted in a slight increase in fecal streptococci count, although the increase was not significant at the 5% level ($P<0.10$). No interaction between ration and terramycin was noted. A significant ration effect ($P<0.05$) however, was observed between the high protein and the basal ration, lower counts obtained with the former. This was the only significant ration effect observed in this study.

Total lactobacilli. The total lactobacilli, enumerated in Rogosa's medium were markedly reduced by addition of terramycin to any of the 3 rations. No interaction obtained between ration and terramycin. Many colonies developing on this medium were found to be streptococci and yeasts, and the counts here reported are from numbers of colonies, without identification, and are considered to be provisional.

---

* This investigation was supported by Chas. Pfizer and Co., Brooklyn, N. Y.

TABLE I. Mean Values of the Logs of All Enumerations of Total Viable Anaerobes, Total Viable Aerobes, *L. acidophilus*, *E. coli*, *Proteus spp.*, Fecal Streptococci and Total Viable Lactobacilli, in the Feces of Albino Rats on 3 Diets with and without Terramycin. Statistical data include ration differences, drug differences, duplication of experiment and significant probabilities.

	Organisms						
Exp. groupings	Total anaerobes count, $\times 10^6$	Total aerobes count $\times 10^6$	*L. acidophilus* count, $\times 10^3$	*Proteus spp.* count, $\times 10^3$	*E. coli* count, $\times 10^4$	Fecal streptococci count, $\times 10^3$	Total lactobacilli count, $\times 10^6$
Rations							
Basal	2.968	2.503	2.595	2.960	2.821	3.624	1.817
Protein	2.847*	2.230	2.378	3.162	2.702*	1.878‡	1.687
Carbohydrate	3.167	2.606	1.945	2.955	3.472	2.739	1.956
Terramycin							
(−)	3.372†	2.685‡	3.334†	1.996†	2.272§	2.389*	2.644†
(+)	2.616	2.207	1.277	4.055	3.724	3.106	1.005
Replication							
I	3.026	2.174‡	2.170	2.782	3.226	2.838	1.812
II	2.962	2.718	2.441	3.269	2.769	2.657	1.837

* P <.10. † P <.001. ‡ P <.05. § P <.01.

L. acidophilus. Terramycin *per se* caused a significant reduction on *L. acidophilus* ($P < 0.05$). No interaction between diet and terramycin was observed, although higher counts were obtained with the basal and the high protein rations than with the high carbohydrate ration. This substantiates the observation that dextrin fails to maintain a high aciduric intestinal flora(1,6,7).

E. coli. Increased dosage of terramycin did not result in a significant interaction between ration and terramycin, as was observed (1) with the low nutritional level. A ration effect, however, was noted at the 10% level of probability, between the high carbohydrate and the high protein diets, higher counts obtained with the former. Terramycin *per se* caused an *increase* ($P < 0.01$) in *E. coli*, an observation in disagreement with the findings of most workers. Attention is particularly directed to the fact that the tergitol-7 agar(8) used in this and prior work(1) has been found to be from 30 to 40% more productive in recovery of *E. coli* than other differential media(9). During the experimental work reported here, hundreds of cultures isolated from this medium were found to give IMViC (10) reactions of "typical" *E. coli.* Although no other studies were made of the organisms recovered, it is believed that the productivity and selectivity of this medium are remarkable, and the counts reliable.

Conclusions. A comparison of this and the previous report is presented in Table III, from which the following conclusions may be drawn: (a) a significant diet and terramycin interaction was observed only in low terramycin dosage, only in counts of *E. coli;* (b) a significant diet effect was observed in *Proteus* counts, only at low dosage, and in counts of fecal streptococci only at high terramycin dosage; (c) significant decreases in total anaerobes, total aerobes, *L. acidophilus,* were found at both dosages; (d) increase of *Proteus* at both dosages; (e) decrease of fecal streptococci at low dosage but slight increase at high dosage. Comparisons illustrate the need for severe analysis of experimental conditions and procedures, and in general indicate validity of the findings.

Summary. 1. In the feces of albino rats, statistically significant reductions in counts

TABLE II. Mean Logs of 5 Weekly Values Including L.S.D. among Subgroups of All Animals on Different Rations with and without Terramycin.

		Basal		High protein, low carbohydrate, terramycin		High carbohydrate, low protein		L.S.D. for subgroups*
Organisms	Exp.	—	+	—	+	—	+	
Total anaerobes ×10^6	I	3.179	2.743	3.502	2.334	3.596	2.800	.398
	II	3.398	2.550	3.254	2.289	3.300	2.970	
	Mean	3.289	2.647	3.378	2.316	3.448	2.886	
Total aerobes ×10^6	I	2.209	2.228	2.259	1.727	2.577	2.045	.640
	II	3.242	2.333	2.898	2.033	2.925	2.878	
	Mean	2.726	2.280	2.579	1.880	2.750	2.461	
L. acidophilus ×10^5	I	3.204	1.021†	3.203	1.320	3.219	1.051	1.193
	II	3.939	2.214	3.292	1.695†	3.149	.357	
	Mean	3.581	1.618	3.248	1.508	3.184	.704	
E. coli ×10^4	I	2.239	4.064	2.120	4.094	2.796†	4.045	.977
	II	1.729†	3.252†	1.772	2.820	2.977	4.069†	
	Mean	1.984	3.658	1.946	3.457	2.886	4.058	
Proteus spp. ×10^3	I	1.672	4.106	2.189	3.337	1.232	4.160	.989
	II	1.852	4.211	2.645	4.477	2.386	4.041	
	Mean	1.762	4.159	2.417	3.907	1.809	4.100	
Fecal streptococci ×10^3	I	2.526	4.492	2.534	1.496	2.756	3.220	1.332
	II	3.236	4.242	1.454	2.123	1.825	3.157	
	Mean	2.881	4.367	1.994	1.762	2.291	3.188	
Total lactobacilli ×10^6	I	2.581	.823	2.547	.702	2.725	1.494†	.794
	II	2.737†	1.185	2.395	1.104	2.879	.724†	
	Mean	2.659	1.004	2.471	.903	2.802	1.109	

* L.S.D. for subgroups computed at 5% level of probability.
† One missing value calculated.

TABLE III. Comparative Statistical Analyses of 2 Sets of Different but Comparable Experimental Data on Effects of Dietary Terramycin on Fecal Microflora.

		Organisms					
	T*	Anaerobes	Aerobes	*L. acidophilus*	*E. coli*	*Proteus spp.*	Fecal streptococci
Terramycin	A	Decrease† ($P < .01$)	Decrease† ($P < .01$)	Decrease† ($P < .01$)	Decrease‡ ($P < .01$) Increase§ ($P < .05$)	Increase‖ ($P < .05$)	Decrease† ($P < .001$)
	B	Decrease† ($P < .001$)	Decrease† ($P < .05$)	Decrease† ($P < .05$)	Increase‖ ($P < .01$)	Increase‖ ($P < .001$)	Increase¶ ($P < .1$)
Diet effect	A	None	None	None	None	Present ($P < .05$)	None
	B	Present¶ ($P < .1$)	”	Present¶	Present¶	None	Present ($P < .05$)**
Diet terramycin interaction	A	None	”	None	” ‡§	”	None
	B	”	”	”	None	”	”

* A = 100 mg terramycin/kg ration. B = 666 mg terramycin/kg ration.
† Decrease in all diets. ‡ Decrease in high carbohydrate ration. § Increase in basal ration.
‖ Increase in all diets. ¶ Not significant. ** Increase in high protein ration.

of the total anaerobes, total aerobes and total lactobacilli were obtained by the addition of terramycin to the 3 diets employed. On the other hand, *E. coli*, *Proteus spp.* and, to a slight extent fecal streptococci, were increased in numbers. The increase in *Proteus* was unaccompanied by a decrease in *E. coli*. 2. The only ration effect was observed with the counts of fecal streptococci: the animals receiving the high protein rations showed a lower count than those receiving either the high carbohydrate or the basal ration. 3. The effect of terramycin upon counts of all organisms studied was found to be independent of ration in which the terramycin was incorporated (lack of statistically significant interaction between diet and terramycin). 4. The effects here reported were compared to the effects observed in a previous study.

Indebtedness is acknowledged to Dr. H. D. Eaton of the Nutritional section, Department of Animal Industries, University of Connecticut, Storrs, for statistical analysis of data.

---

1. Scaletti, J. V., Kulp, W. L., Cleverdon, R. C., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 552.
2. Sieburth, J. Mc., Gutierrez, J., McGinnis, J., Stern, J. R., and Schneider, B. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 15.
3. Sieburth, J. Mc., McGinnis, J., and Skinner, C. E., *J. Bact.*, 1952, v64, 163.
4. Rogosa, M., Mitchell, J. A., Wiseman, R. F., *J. Bact.*, 1951, v62, 132.
5. Snedecor, G. W., *The Iowa State College Press*, Ames, Iowa, 1946, 268.
6. Nath, H., Barki, V. H., Sarles, W. B., and Elvehjem, C. A., *J. Bact.*, 1948, v56, 783.
7. Johansson K. R., Shapiro, S. K., and Sarles, W. B., *J. Bact.*, 1948, v56, 619.
8. Chapman, G. H., *Am. J. Pub. Health*, 1951, v41, 1381.
9. Kulp, W. L., Mascoli, C., and Tavshanjian, O., *Am. J. Pub. Health*, 1953, in press.
10. Parr, L. W., *Am. J. Pub. Health*, 1936, v26, 39.

Received June 10, 1953. P.S.E.B.M., 1953, v83.

Failure of Certain Vitamins to Affect the Survival of the Eviscerate Rat. (20431)

DWIGHT J. INGLE, JAMES E. NEZAMIS AND ERVING H. MORLEY.

From the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

It was considered possible that vitamin deficiencies might ensue rapidly in the eviscerate animal and thereby limit survival. Eviscerate rats were injected with different preparations of vitamins without a favorable effect upon survival.

Methods. Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets. The technic of aseptic evisceration has been described(1). When the animals reached 250 ± 2 g, they were anesthetized (intraperitoneal injection of 18 mg of cyclopal sodium) and subjected to the second stage of evisceration. Sterile solutions containing 0.9% NaCl, glucose, insulin, crystalline penicillin G, and streptomycin sulfate with and without vitamins were administered by a 6-place continuous injection machine which delivered fluid into the saphenous vein of the right hind leg at the rate of 20 ml per 24 hours. The initial glucose load was 44 mg per 100 g of rat per hour and was decreased to 36 mg after 48 hours. The insulin dose was 4 units per rat per 24 hours. The dose of penicillin was 10,000 units per rat per 24 hours, and the streptomycin dose was 20 mg per rat per 24 hours. Temperature was 26.5 ± 0.5°C. Survival time was determined by a device which amplified the D.C. potential generated by the heart to actuate a recording mechanism.

The following vitamins were used. A mixture of B vitamins contained the following amounts per ml: Vit. B_{12}, 1 μg; folic acid, 1 mg; thiamine hydrochloride, 2 mg; riboflavin, 2 mg; pyridoxine hydrochloride, 1 mg; pantothenate sodium, 10 mg; and nicotinamide, 25 mg. Rutin and ascorbic acid were mixed together in a 1 to 1 weight ratio. The above substances were added to the solutions given by continuous intravenous injection. The oil soluble vit. A, D, and E were given together by continuous subcutaneous injection at the rate of 1 ml per rat per 24 hours. Cod liver oil was the source of vit. A and D; mixed tocopherols were added.

Experiments and results. Eviscerate rats given vitamins were compared simultaneously with eviscerate rats not given vitamins but

TABLE I. Survival of Eviscerate Rats with and without the Administration of Vitamins.

	Min survival					
	Vitamins			No vitamins		
Vit dose/24 hr/rat	N	Avg	Range	N	Avg	Range
ml						
B-complex .05	10	6102	4491-7310	11	6202	5169-7511
.125	12	6367	5441-7447	12	6424	5914-7528
.25	12	5540	4792-6566	12	5753	4670-7226
.50	12	5804	4538-6761	12	6167	5010-7178
Rutin 1 mg; ascorbic acid 1 mg	15	5938	4717-7631	14	5955	4514-7493
" 2 "; " " 2 "	10	6279	5078-6940	11	6193	5221-7759
A— 80 u; D—12 u; E—4 mg	12	5749	5256-6805	11	5892	4680-6875
A—160 u; D—24 u; E—8 mg	10	5537	4214-6925	10	5687	4894-6493

otherwise treated in an identical manner. The data on treatment, number of rats per group and times of survival are summarized in Table I. The results were negative.

Discussion. These data do not exclude the possibility that other vitamins or combination of vitamins not tested here would affect the survival of the eviscerate rat. It has been suggested to us that exogenous vitamins may be unable to act in the eviscerate rat without the addition of their coenzymes. The mitochondria fraction of rat liver extracts has been added to exogenous vitamins in a few animals on the assumption that this would provide the coenzymes of vitamins but the results of these preliminary tests were negative. Several extracts of beef liver have been tested in the eviscerate rat without a favorable effect upon survival. One of the control eviscerate rats of this series survived for 138 hours, the longest survival of any eviscerate mammal of which we are aware.

Summary. Male eviscerate rats were fed intravenously with solutions of 0.9% NaCl, glucose, insulin, streptomycin, and penicillin with and without the following mixtures of vitamins: B_{12}, folic acid, thiamine, riboflavin, pyridoxine, pantothenate and nicotinamide; rutin and ascorbic acid; and vit. A, D, and E. The results were negative.

---

1. Ingle, D. J., *Exp. Med. Surg.*, 1949, v7, 34.

Received June 15, 1953. P.S.E.B.M., 1953, v83.

Effect of Phenylbutazone on Uric Acid Excretion in Patients with Gout and Rheumatoid Arthritis. (20432)

CHARLES BISHOP AND LAWRENCE BEECHER. (Introduced by Fred R. Griffith, Jr.)

From the Chronic Disease Research Institute, Buffalo, N. Y.

Phenylbutazone* (3,5 dioxo-1,2-diphenyl-4-n butylpyrizolidine sodium) is one of the newer drugs used in the treatment of gout and rheumatoid arthritis. Although phenylbutazone is not a steroid it seems to promote a sense of well-being in certain gouty and arthritic patients, over and above its simple analgesic action(1).

The experience in both this and other clinics is that the institution of phenylbutazone therapy is usually followed by a noticeable decrease in the serum urate level. Studies have been undertaken in this laboratory to see if this effect can be explained on the basis of simple uricosuric action. A preliminary study is reported in this paper.

Methods. Two patients with gout and 3 with rheumatoid arthritis were hospitalized. They consumed a diet uniformly low in purines and constant in nitrogen and caloric content.

---

* Butazolidin kindly supplied by Geigy Pharmaceuticals Division of the Geigy Co., New York City.

TABLE I. Urinary Uric Acid Output after Phenylbutazone Therapy.

Patient, Sex	Age, diagnosis†	Therapy*	No. of days	Mean daily uric acid excretion (mg)	Stand. dev. (mg)	T test	P value	Serum urate
W, ♀	64, RA	C	7	311	53			4.7, 4.9
		C + P	7	355	69	$T_{12} = 1.34$	$P > 10\%$	4.4, 3.2
		P	7	354	35	$T_{13} = 1.77$	"	2.6, 2.1, 2.5
B, ♀	54, RA	C	7	359	49			4.0, 4.5, 3.9
		C + P	7	415	66	$T_{12} = 1.64$	"	3.8, 0.9, 1.3
		P	7	390	85	$T_{13} = 1.09$	"	1.3, 1.8
H, ♀	50, RA	0	7	446	45			6.4, 6.1, 5.6
		P	11	513	92	$T_{12} = 1.65$	"	4.8, 4.4, 3.2
		0	10	404	93	$T_{13} = 1.13$	"	4.0, 7.5, 7.9
Q, ♂	82, NG	0	7	296	65			9.2, 8.5, 9.4
		C + P	7	374	61	$T_{12} = 2.50$	$5\% > P > 2.5\%$	9.4, 7.7, 7.9
		P	14	374	52	$T_{13} = 3.03$	$1\% > P > 0.5\%$	7.0, 11.0, 7.3, 4.9
		0	7	368	104	$T_{14} = 1.60$	$P > 10\%$	4.5, 5.8, 5.6
E, ♂	72, TG	0	7	398	52			11.3, 12.9
		B	7	627	136	$T_{12} = 4.12$	$P < 0.5\%$	10.5, 11.0
		P	8	514	121	$T_{13} = 2.27$	$5\% > P > 2.5\%$	11.4, 11.1, 10.0
		B + P	7	618	83	$T_{14} = 5.58$	$P < 0.5\%$	7.6, 7.7

* C = Codeine (P.R.N., not more than 1 grain daily). P = Phenylbutazone (200 mg 2 times daily). B = Benemid (500 mg 4 times daily).

† RA = Rheumatoid arthritis. NG = Non-tophaceous gout. TG = Tophaceous gout.

During the entire study all urine was collected daily and the uric acid output was determined using the method of Folin(2). After a control period, phenylbutazone therapy was instituted (2 x 200 mg daily). During the control period two of the arthritic patients had been receiving codeine (P.R.N., not more than 1 grain daily) to control their pain and this was continued for the first period of phenylbutazone therapy. In these cases a second period of phenylbutazone therapy was instituted during which the codeine was eliminated and only the phenylbutazone was given. A final control period was used wherever practical. In one of the gouty patients, Benemid† (p-(di n-propyl sulfamyl) benzoic acid) and phenylbutazone were administered both separately and together in order to compare the action of phenylbutazone with that of a known uricosuric agent(3). The details of the regimens and the results are summarized in Table I.

Discussion. It is apparent from Table I that phenylbutazone caused a lowering of the serum urate level in every subject except S.E. Phenylbutazone appeared to cause a small increase in uric acid excretion in the three arthritic patients but the increase was not statistically significant when the data were tested by the t test. With the gouty patients, however, the increase was unequivocal. Thus it would appear that at least in certain situations, phenylbutazone acts as a uricosuric agent. Whether this is its only mechanism of action for lowering the serum uric acid concentration cannot be deduced from this type of study.

Since phenylbutazone does have a uricosuric action, it may be difficult by means of balance studies to show that the uric acid that disappears from the serum does or does not equal the "extra" uric acid excreted in the urine. The difficulties of attempting to equate such factors have already been discussed for the drug Benemid(3).

Summary. Phenylbutazone was shown to cause a statistically significant increase in urinary uric acid output in two gouty subjects. Such an increase could not be demonstrated in three patients with rheumatoid arthritis.

---

† Benemid, kindly supplied through the courtesy of Sharpe and Dohme, Philadelphia, Pa.

1. Essential Clinical Data on Butazolidin, Geigy Pharmaceuticals Division of Geigy Company, Inc., 220 Church Street, New York City 13.

2. Folin, O., *J. Biol. Chem.*, 1933, v101, 111; 1934, v106, 311.

3. Bishop, C., Rand, R., and Talbott, J. H., *J. Clin. Invest.*, 1951, v30, 889.

Received June 15, 1953. P.S.E.B.M., 1953, v83.

Antibiotic Combinations and Resistance to Antibiotics: Penicillin with Other Antibiotics Against Penicillin-Resistant Staphylococci.* (20433)

MAXWELL FINLAND AND CLARE WILCOX.

From the Thorndike Memorial Laboratory, 2nd and 4th Medical Services, Boston City Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass.

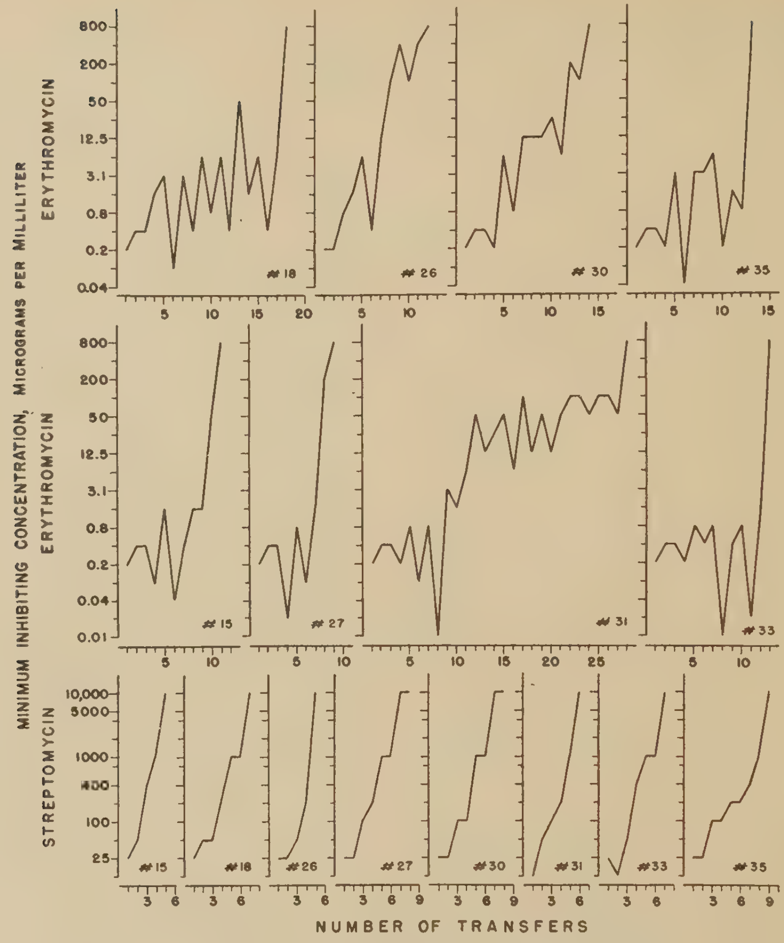
In a previous study(1) it was shown that the development of resistance to penicillin, streptomycin or erythromycin *in vitro* may be delayed and depressed by the use of a combination of erythromycin with either penicillin or streptomycin. The organisms used in that study were originally susceptible to small or moderate concentrations of each of these antibiotics, and when they were repeatedly subcultured in the combination of 2 antibiotics, these were present in increasing concentrations, but always in a constant ratio. In the present study an attempt was made to determine whether the presence of a moderately high concentration of an antibiotic to which bacteria are originally resistant would also serve to delay or depress the development of resistance to another antibiotic following repeated exposures to increasing concentrations of the latter.

Materials and methods. The 8 strains of *Staphylococcus aureus* used in the present study had been isolated from infected materials during a recent survey(2); they were each inhibited by 25 μg/ml of streptomycin and 0.2 μg/ml of erythromycin but grew well in the presence of 1000 μg/ml of penicillin. They were all hemolytic, coagulase-positive and produced penicillinase. Each of the strains was subcultured on 3 parallel series of blood agar plates: one contained no antibiotic, the second contained penicillin 200 μg/ml plus graded (2-fold) concentrations of streptomycin [as in the plate-dilution test for sensitivity(3)], while the third contained the same amounts of penicillin and similarly graded concentrations of erythromycin. The inoculum for the antibiotic-containing plates was obtained in each instance by scraping a 3 mm loopful of the surface growth from the plate containing the maximum concentration of antibiotic on which nearly full growth occurred after 48 hours incubation, suspending this in 0.2 ml of broth and streaking a 1 mm loopful of the suspension on a similar series of plates. This method is similar to the one employed in previous studies(1,4-8). At the completion of 25 such subcultures (except strain 31, which was subcultured 29 times) all of the resulting strains were tested simultaneously for sensitivity to penicillin, streptomycin, erythromycin, carbomycin and neomycin by the usual plate-dilution method(3). Sensitivity of any strain to a given antibiotic is defined as the minimum concentration of that antibiotic in which there is no visible growth after 48 hours.

Results. Resistance to streptomycin and erythromycin. The changes in the sensitivity of the strains during successive transfers in the presence of a constant concentration of penicillin and increasing concentrations of either streptomycin or erythromycin are depicted in Fig. 1. The development of resistance to streptomycin in the presence of penicillin was uniformly rapid and was virtually complete after from 5 to 8 transfers. Resistance to erythromycin under similar circumstances developed somewhat more irregularly,

---

* Aided by a grant from the U. S. Public Health Service. The erythromycin was supplied by Dr. J. W. Smith of Lilly Research Laboratories, the Carbomycin (Magnamycin) by Dr. Gladys L. Hobby of Chas. Pfizer and Co. and neomycin by Dr. Earl L. Burbidge of Upjohn Co.



CONCENTRATION OF PENICILLIN CONSTANT, 200 MICROGRAMS PER ML.

FIG. 1.

but each of the strains had become resistant to 800 μg/ml after from 9 to 18 subcultures, except in the case of Strain 31 which required 28 transfers before this degree of resistance was acquired.

Cross-resistance. It was previously shown that high degrees of cross-resistance to carbomycin quite regularly accompanied the development of resistance to erythromycin following exposures to the latter *in vitro*(8). Cross-resistance between streptomycin and neomycin have also been demonstrated although less regularly(6). In the present study, the sensitivity of all of the strains after completion of the 3 series of transfers was tested simultaneously to the 3 antibiotics used in the study and also to carbomycin and neomycin. The results are shown in Table I. No cross-resistance developed to erythromycin following the subcultures in streptomycin, and *vice versa*. Resistance to carbomycin developed to a high degree in each of the strains as a result of the exposures to erythromycin. A significant increase in resistance to neomycin occurred in only 1 strain and a slight increase to another at the end of the series of subcultures on streptomycin. One of the strains (No. 32) became streptomycin-dependent during the course of the exposures to streptomycin; none of the other 4 antibiotics (including neomycin) in any of a wide range of concentrations could support the growth of this streptomycin-dependent strain in the absence of streptomycin. All strains of the streptomy-

TABLE I. Effect of Repeated Subcultures of Penicillin-Resistant Staphylococci on Agar Containing Constant Concentration of Penicillin (200 μg/ml) plus Graded Concentrations of Streptomycin or Erythromycin on Their Resistance to Each of These Antibiotics and also to Carbomycin and Neomycin.

Strain	Subcultures*	Minimum inhibiting concentration, μg/ml				
		P	S†	E†	C	N
15	0 × 25	>6400	25	.4	12.5	1.6
	P + S × 25	>6400	>10000 (5)	.2	3.1	1.6
	P + E × 25	>6400	25	>800 (11)	>800	.8
18	0 × 25	6400	25	.4	12.5	1.6
	P + S × 25	800	>10000 (7)	.2	3.1	1.6
	P + E × 25	>6400	25	>800 (18)	400	1.6
26	0 × 25	6400	25	.4	12.5	1.6
	P + S × 25	800	>10000 (5)	.2	.8	3.1
	P + E × 25	6400	25	>800 (12)	400	.4
27	0 × 25	6400	25	.4	12.5	.8
	P + S × 25	800	>10000 (8)	.2	3.1	3.1
	P + E × 25	6400	12.5	>800 (9)	400	.8
30	0 × 25	>6400	25	.4	12.5	1.6
	P + S × 25	>6400	>10000 (8)	.2	3.1	3.1
	P + E × 25	>6400	25	>800 (14)	800	.8
31	0 × 29	1600	25	.4	12.5	1.6
	P + S × 29	1600	>10000 (6)	.2	3.1	6.3
	P + E × 29	3200	12.5	>800 (28)	200	3.1
32	0 × 25	>6400	25	.4	12.5	1.6
	P + S × 25	S-Dep.‡	>10000 (7)	S-Dep.	S-Dep.	S-Dep.
	P + E × 25	6400	12.5	>800 (13)	>800	1.6
35	0 × 25	>6400	25	.4	12.5	1.6
	P + S × 25	3200	>10000 (9)	.2	6.3	25
	P + E × 25	6400	12.5	>800 (14)	>800	1.6

P = penicillin; S = streptomycin; E = erythromycin; C = carbomycin; N = neomycin.

* 0 = No antibiotic; concentration of penicillin in mixtures was constant (200 μg/ml).

† Number of transfers required to achieve maximum concentration is shown in parentheses. The organisms at start of experiment were each inhibited by streptomycin, 25 μg/ml and by erythromycin, 0.2 μg/ml.

‡ Streptomycin-dependent.

cin series became somewhat more sensitive to carbomycin, but their neomycin sensitivity was not affected by the exposures to erythromycin.

Effect on coagulase and penicillinase. Each of the strains was tested for coagulase activity on several occasions during the course of the study. For the most part, coagulase was demonstrated in all of the strains throughout the study; some irregularities were noted, however, on a few strains at times when it was necessary to resort to the use of plasma from different donors for the tests. All of the parent strains produced penicillinase(9) and this property was retained essentially intact throughout the 3 series of subcultures.

Summary and conclusion. Eight strains of staphylococci which were originally resistant to penicillin but sensitive to streptomycin and erythromycin were repeatedly subcultured in the presence of 200 μg/ml of penicillin along with increasing concentrations of one of the other 2 antibiotics. Resistance to streptomycin or erythromycin resulted from the exposures to the homologous agent in spite of the presence of the high but ineffective concentration of penicillin. Cross-resistance between streptomycin and erythromycin did not occur, but high degrees of resistance to carbomycin were demonstrated in all of the strains which had become resistant to erythromycin and some increase in resistance to neomycin occurred in 2 of the strains which had become resistant to streptomycin. These findings suggest that for the suppression or delay in development of resistance to one of a pair of antibiotics to which bacteria are simultaneously exposed, both of the antibiotics must exert an inhibitory activity; if the strain is resistant to one of the pair of antibiotics, its presence in a high but ineffective concentration may not influence the development of resistance to the second agent.

1. Purcell, E. M., Wright, S. S., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 124.
2. Finland, M., and Haight, T. H., *Arch. Int. Med.*, 1953, v91, 143.
3. Jackson, G. G., and Finland, M., *Arch. Int. Med.*, 1951, v88, 446.
4. Murray, R., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 470.
5. Gocke, T. M., and Finland, M., *J. Lab. and Clin. Med.*, 1951, v38, 719.
6. Haight, T. H., Wilcox, C., and Finland, M., *J. Lab. and Clin. Med.*, 1952, v39, 637.
7. Haight, T. H., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 183.
8. Finland, M., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 725.
9. Haight, T. H., and Finland, M., *Am. J. Clin. Path.*, 1952, v22, 806.

Received June 17, 1953. P.S.E.B.M., 1953, v83.

Fatty Acid and Ketone Metabolism during Hemorrhage and Shock in the Rat.[*] (20434)

FRANK L. ENGEL AND KATHLEEN HEWSON.
From the Departments of Medicine and Physiology, Duke University, Durham, N. C.

Although there is now available considerable information concerning protein and carbohydrate metabolism during hemorrhagic shock in the rat, little or nothing is known about fat metabolism in this syndrome(1-3). Since hepatic metabolism appears to be particularly disturbed during shock, it seemed pertinent to investigate some aspects of ketone body metabolism since the liver is the chief site of synthesis of these substances. It has been shown that the oxygen consumption and the rates of a variety of oxidative reactions by liver slices from shocked rats may be profoundly depressed, but the degree of depression of oxygen consumption cannot be accounted for by the metabolic disturbances so far demonstrated in the liver. Since, during fasting, fat oxidation and ketone production may account for a very substantial part of the oxygen requirements of the liver, this study was initiated in an attempt to determine whether fatty acid and ketone metabolism might be sufficiently disturbed during hemorrhage and shock in the rat to contribute to the observed lowered hepatic oxygen consumption.

Materials and methods. Male albino rats of the Vanderbilt strain, weighing between 200 and 270 g, were fasted 24 hours prior to bleeding. Shock was induced under light nembutal anesthesia by bleeding from the cut tail a volume of blood equivalent to 2.5% of the body weight in one hour. Blood samples for ketone and amino nitrogen determinations were withdrawn at appropriate intervals, and the animals observed for 3 to 5 hours and then sacrificed for other studies(4,5). In one group of rats, adrenalectomies were performed 4 to 5 days prior to bleeding, the animals being maintained with 0.5 mg of desoxycorticosterone acetate intramuscularly daily and saline in their drinking bottles. Ketone levels were determined on 0.4 ml samples of heparinized whole blood by a modification of the method of Greenberg and Lester(6,7) and amino nitrogen in plasma by the method of Frame *et al.*(8).

Results. The effects of hemorrhage and shock on blood ketone levels are depicted in Fig. 1. The control rats, which had blood samples drawn just for analyses every hour for 7 hours, exhibited a slow progressive rise in ketone levels, as might be anticipated during a continuing fast. The bled rats were divided into 2 groups, designated as "sublethal hemorrhage" and "hemorrhagic shock"

---

[*] This work was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army and the American Cancer Society, administered by the Committee on Growth, the National Research Council.

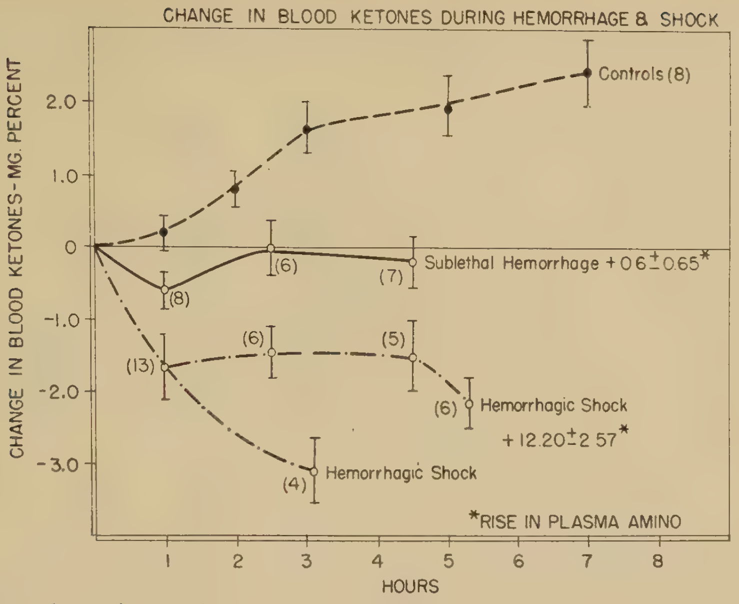


FIG. 1. Mean change in mg % ± S.E. in blood ketone levels in rats subjected to hemorrhage and shock. The numbers in parentheses refer to the number of observations at each point.

in terms of their clinical condition and degree of elevation in plasma amino nitrogen. Previous studies have demonstrated that the rate and degree of rise in plasma amino nitrogen following hemorrhage is a reliable indication of the severity of shock in the rat(9,10). On the basis of these data, all animals which had shown less than 3 mg % rise in amino nitrogen 3 to 4 hours after bleeding were classified as "sublethal hemorrhage", while those with greater than 3 mg % rise were considered to be in irreversible shock. Actual survival could not be determined since the animals were sacrificed at the end of the experiment for other studies. Note that the "sublethal hemorrhage" group had a mean plasma amino nitrogen elevation of 0.6 ± 0.65[†] mg % and that the usual rise in blood ketone levels was abolished. The hemorrhagic shock animals were divided into 2 groups in terms of their being obviously preterminal at approximately 3 and 5½ hours, respectively. In both groups, there was a very significant depression of ketone levels, the degree of depression varying with the rapidity with which shock developed. Plasma amino nitrogen levels rose progressively, with the mean final levels for all animals being +12.20 ± 2.57 mg %.

In a group of 6 adrenalectomized rats bled 2.0 and 2.5% of body weight, essentially similar results were found. Ketone levels dropped 1.21 ± 0.45 mg % at the end of bleeding and 2.16 ± 0.47 mg % by 3½ hours, plasma amino nitrogen increasing by 9.50 ± 2.08 mg %.

In an attempt to seek an explanation for the hypoketonemia of hemorrhage and shock, the effect of an intravenous infusion of sodium octanoate on ketone levels during hemorrhage and shock was investigated. Sodium octanoate, a fatty acid which is a well known ketone precursor, was infused over a 30-minute period into the external saphenous vein in a dose of 1.5 ml of a sterile 2% solution per 100 g body weight, beginning 2½ hours after bleeding the rats 2.5% of their body weight. The results are seen in Fig. 2, where they are plotted as mean change in blood ketone levels from the beginning to the end of the infusion. Ketone levels at the end of the infusion in normal animals increased by 4.40 ± 0.16 mg %, infusion of an equal volume of saline having no measurable effect. The ketone levels reach their peak at the end of the infusion and thereafter rapidly decline. In rats in mild shock,

---

[†] All values expressed as mean ± standard error.

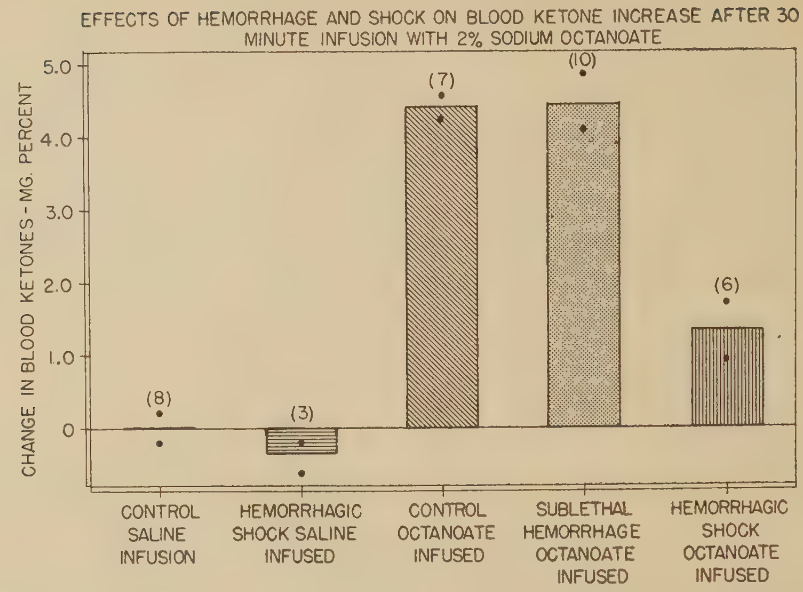


FIG. 2. Mean change in mg % ± S.E. in blood ketone levels during a 30-min. intravenous infusion of 1.5 ml of 2% sodium octanoate per 100 g body wt in normal and bled rats.

as indicated by a mean rise in plasma amino nitrogen of only 1.02 ± 0.20 mg %, the response to octanoate infusion was identical with the controls, *i.e.*, a rise of 4.45 ± 0.43 mg % in 30 minutes. In contrast, rats in severe shock, with amino nitrogen elevations of 6.08 ± 1.77 mg % prior to infusion, exhibited a significantly smaller rise in ketones after octanoate infusion, amounting to 1.35 ± 0.46 mg %. Compared with the sublethal hemorrhage group, these animals tolerated the infusion poorly, usually dying shortly after its termination with amino nitrogen levels rising to 13.81 ± 3.16 mg % above the starting levels. Comparable shocked rats, receiving a similar volume of saline, showed an insignificant fall in blood ketones. In this group, plasma amino nitrogen had risen 4.05 ± 0.16% prior to infusion and 8.95 ± 2.18 mg % at the end of the infusion, with death following shortly thereafter. Blood ketone levels exhibited the same decline in 2½ hours after hemorrhage and during shock as in the previous series.

Discussion. The most striking finding of this study was the ease with which blood ketone levels were depressed in the rat by a hemorrhage which was not sufficient to produce clinical evidences of shock or elevate plasma amino nitrogen levels. Aside from the early rise in blood sugar, attributable to epinephrine discharge, and the initial fall in plasma amino nitrogen, occurring immediately after bleeding(2), no other metabolic responses to hemorrhage have been recorded as appearing so promptly. This high sensitivity of blood ketone levels to minor disturbances in the circulation must be reckoned with in any study of ketonemia in the rat. Our experience suggests that it may be a factor of importance in any studies on ketosis in the adrenalectomized rat, since this preparation is very likely to be suffering from incipient circulatory failure(11).

The mechanism of the hypoketonemia of hemorrhage and shock is not immediately apparent from these studies. Since it occurs with equal rapidity in the absence of the adrenal glands, epinephrine discharge cannot be held responsible, even though epinephrine injection may cause a transitory depression followed by a rise in blood ketone levels in normal rats(11). By the same token, adrenal cortical discharge can be eliminated as a factor, although cortisone treatment suppresses both fasting and stress ketosis(11,12).

The lowered ketone levels might be attributed to either a depressed hepatic production of ketone bodies or an accelerated utilization by the peripheral tissues, or both. The data on octanoate infusion in "sublethal" hemorrhage suggest that ketone production from an exogenous fatty acid is not disturbed under these conditions, but do not throw any light on possible changes in the rates of fat catabolism or ketone production from endogenous fatty acids in the liver or on possible alterations in the rate of ketone utilization. As shock progresses, however, suggestive evidence for an impairment in hepatic ketone production from infused octanoate becomes apparent in the lesser rise in ketone levels. This could be due to either decreased delivery of octanoate to the liver or to impaired oxidation of fatty acids by the liver. Since the oxygen supply to the liver apparently decreases as shock progresses(13) and since fat oxidation and ketone production demand a considerable amount of oxygen, a suppression of ketone production from fatty acids by the liver during shock would not be unexpected.

Less is known about the possibility that ketone utilization might be increased during hemorrhage and shock. Further studies are planned on the rate of disappearance of ketone bodies infused into normal and hepatectomized rats during hemorrhage and shock.

The present observations were made on rats under nembutal anesthesia with body temperature maintained by exposure to the heat of an electric lamp. Recent studies have indicated that under these conditions ketosis is greater during fasting in normal rats than in unanesthetized rats not exposed to an external source of heat(11). Further observations are necessary to demonstrate whether the above results still apply to unanesthetized rats subjected to hemorrhage. Preliminary data suggest that they do.

Summary. 1. Sublethal hemorrhage in the normal fasted rat without a significant rise in plasma amino nitrogen is followed by a prompt suppression of fasting ketosis but is without effect on the increase in blood ketones consequent to a 30-minute intravenous infusion of sodium octanoate. 2. Hemorrhagic shock with rising plasma amino nitrogen levels is associated with a significant and progressive hypoketonemia and an inhibition of the rise in blood ketones ordinarily seen after sodium octanoate infusion. 3. Adrenalectomized rats exhibit the same decline in blood ketone levels during hemorrhage and shock as do normal rats. 4. The significance of these results with respect to effects of hemorrhage and shock on ketone production and utilization in the rat is discussed.

We are indebted to Dr. L. Kinsell, Oakland, Calif., and to Dr. J. O'Donovan of the Armour Laboratories, Chicago, Ill., for generous supplies of sterile sodium octanoate solution.

---

1. Wilhelmi, A. E., *Ann. Rev. Physiol.*, 1948, v10, 259.

2. Engel, F. L., *Ann. N. Y. Acad. Sci.*, 1952, v55, 381.

3. ———, *Proc. 2nd Conf. on Shock and Circulatory Homeostasis.* Josiah Macy, Jr. Foundation, New York, N. Y., 1953, in press.

4. Schwartz, T. B., PROC. SOC. EXP. BIOL. AND MED., in press.

5. Schwartz, T. B., and Engel, F. L., PROC. SOC. EXP. BIOL. AND MED., in press.

6. Greenberg, L. A., and Lester, D., *J. Biol. Chem.*, 1944, v154, 177.

7. Lester, D., and Greenberg, L. A., *J. Biol. Chem.*, 1948, v174, 903.

8. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, 1943, v149, 225.

9. Engel, F. L., Winton, M. G., and Long, C. N. H., *J. Exp. Med.*, 1943, v77, 397.

10. Sayers, M. A., Sayers, G., Engel, M. G., Engel, F. L., and Long, C. N. H., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 20.

11. Engel, F. L., and Engel, M. G., unpublished observations.

12. Scott, J. L., Jr., and Engel, F. L., *Endocrinology*, 1953, v53, ——.

13. Engel, F. L., Harrison, H. C., and Long, C. N. H., *J. Exp. Med.*, 1944, v79, 9.

Received June 18, 1953. P.S.E.B.M., 1953, v83.

Production of Fever by Influenzal Viruses. IV. Tolerance to Mumps and Influenza Pyrogens. (20435)

ROBERT R. WAGNER.

From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.

Intravenous injection of influenza or Newcastle disease viruses produces a characteristic febrile response in the rabbit(1). Animals are resistant to a second injection of homologous virus 24 hours later even if the initial febrile reaction is blocked by the administration of antipyrine(2). Resistance to virus toxic action can also be demonstrated for heterologous strains but the degree of tolerance is somewhat more variable under these conditions. In the earlier papers in this series, it was suggested that this nonreactive state to a second injection of virus was correlated with the receptor-destroying activity of the virus strain used for the initial injection.

Recently, French(3) has demonstrated that tolerance to the fever-producing effects of heterologous strains is dependent upon the dose of the blocking virus. The experiments reported here with the PR8 strain of influenza A and mumps virus support French's observations.

Materials and methods. Viruses. Allantoic fluid suspensions of the 151st egg passage of the PR8 strain of influenza A virus and the 161st egg passage of the Enders strain of mumps virus were used. Fluids containing the PR8 virus were aspirated from 12-day-old chick embryos which had been infected 44 hours previously with a 10^{-3} dilution of passage material. The mumps allantoic fluids were obtained from 13-day-old embryos 5 days after intra-allantoic injection with a 10^{-3} dilution of seed inoculum. The individual virus suspensions were pooled and stored in glass-sealed ampoules at –60°C for future use. The fluids were found to be free of bacteria by culture in thioglycollate broth. All glassware was baked at 170°C to prevent contamination with bacterial pyrogens. Hemagglutination titrations were done by a modification of the Salk method(4) using 0.5% chicken erythrocytes. The hemagglutinin titer of the PR8 virus preparation was 1920 and the mumps allantoic fluid, 960.

Rabbits. Male New Zealand rabbits weighing 2-3 kg were used. The experimental procedure was identical to that previously described(1). The usual duration of a single experiment was 7 hours during which time the animals were immobilized by tight-fitting neck-bands. Temperatures were recorded at ½- or 1-hour intervals for 6 hours after the intravenous injection of the virus suspension. Three temperatures were recorded prior to the injection of virus to establish a base line.

Results. Response to a single injection of mumps virus. Six animals in each of 3 groups were injected intravenously with 1, 2 or 5 ml of the undiluted mumps virus suspension. All 18 animals showed a uniform type of febrile response irrespective of the virus dose. In general, a slight elevation of temperature above the base line was noted 1½ to 2 hours after injection. The peak response of 3-4°F occurred between the 3rd and 5th hours followed by a gradual return to the preinjection base line level. Most of the rabbits had normal or only slightly elevated temperatures 24 hours after the start of the experiment. Control animals receiving normal allantoic fluid from 13-day-old chick embryos remained afebrile. The average temperature responses for each dose of virus are shown in Fig. 1. These curves are strikingly similar in appearance to those previously demonstrated for influenza viruses(1).

Tolerance to the mumps pyrogen. Twenty-four hours after the initial injection, the animals which had received 1 and 5 ml of mumps virus were challenged with 1 ml of the homologous virus. Six animals which had previously been injected with normal allantoic fluid served as controls. This last group responded in typical fashion to the mumps virus injection. On the other hand previous exposure to a 1 ml dose of the virus produced a state of partial resistance; the onset of the fever was delayed and the height of the maximal elevation was significantly lower than the controls.

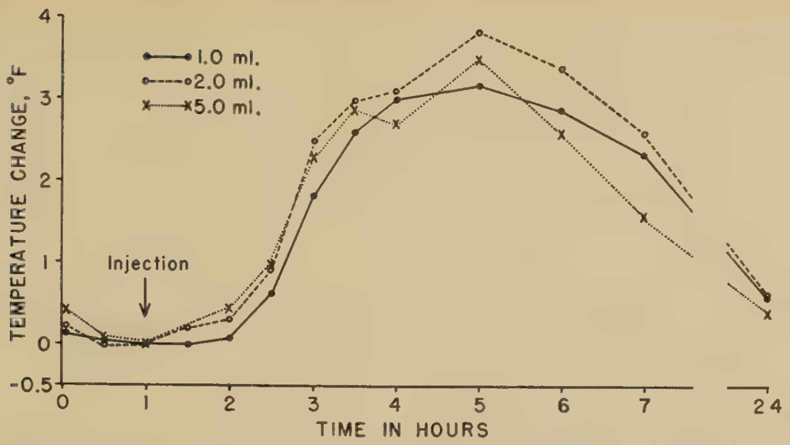


FIG. 1. Febrile responses in rabbits to various doses of a single intravenous injection of mumps allantoic fluid. Each point on the curves represents mean temperature of 6 rabbits.

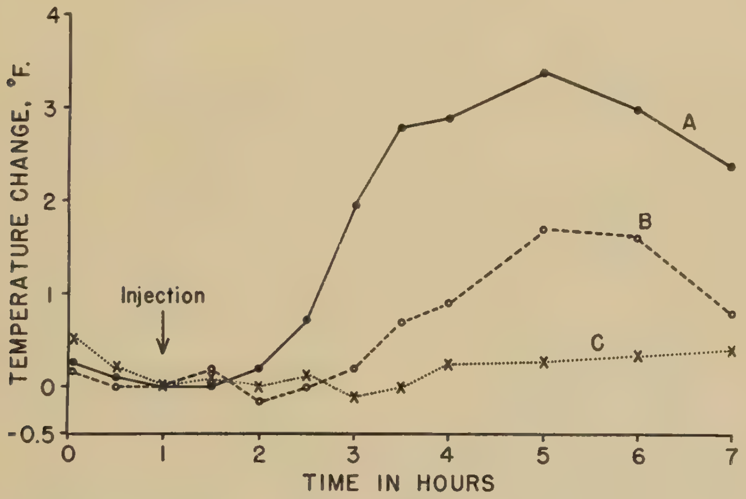


FIG. 2. Temperature responses of groups of 6 rabbits to 1 ml of mumps virus 24 hr after intravenous injection of A. normal allantoic fluid, B. 1 ml mumps virus, C. 5 ml mumps virus.

In the group pretreated with 5 ml of the mumps virus suspension the capacity to respond to a repeat injection was completely blocked (Fig. 2). It is apparent from these results that the degree of resistance induced by the first injection of virus is independent of the magnitude of the initial febrile response. As shown in Fig. 1 the temperature elevations following injection of 1 or 5 ml of virus in normal animals are in the same range. Nevertheless the 5 ml dose of the virus suspension afforded more effective protection to subsequent challenge.

Tolerance to the PR8 pyrogen. The presence of a refractory state to a repeat injection of PR8 virus(2) was readily confirmed. Six animals responded to an intravenous injection of 1 ml of PR8 virus with febrile reactions of a slightly lower order but which were otherwise quite similar to those following mumps virus injection. A second injection of the same dose of PR8 virus 24 hours later was completely without effect. The only apparent difference in the tolerance-producing action of these two agents is the more solid resistance to the homologous strain after a smaller dose of PR8 virus(2).

Cross tolerance between mumps and influenza viruses. The fever-producing capacities of mumps and PR8 viruses were studied in

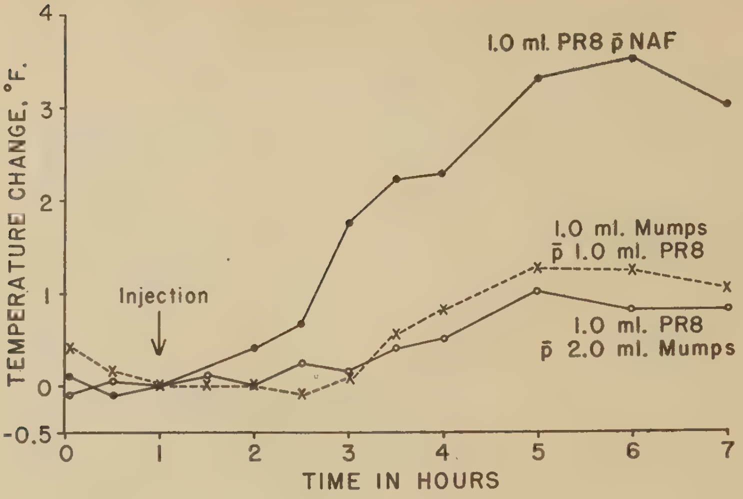


FIG. 3. Tolerance to heterologous virus challenge. Temperature records are for the second virus injection in groups of 6 animals.

animals pretreated with the heterologous strain. Six animals were prepared by intravenous injection of 2 ml of mumps virus and another group of 6 received 1 ml of PR8 virus. Each of the 12 animals, therefore, was pretreated with 7,680 hemagglutinating units of the respective virus preparations. Twenty-four hours later the mumps-treated animals were challenged with 1 ml of PR8 virus and the PR8-treated animals with 1 ml of mumps virus. The mean temperature curves for the second injection of virus are shown in Fig. 3. For comparison the average response in 6 rabbits to an injection of PR8 virus 24 hours after an injection of normal allantoic fluid is also presented in Fig. 3. It is readily apparent that a refractory state develops to heterologous virus challenge. The resistance was not complete but was of the same degree in each cross tolerance experiment.

Discussion. A property shared by all viruses of the influenza group is their capacity to destroy virus receptors on erythrocyte and other cell surfaces (influenza C is an obvious exception). However certain strains are more effective receptor-destroying agents than others. By testing the capacity of a standard dose of 1 strain to remove the erythrocyte receptors for other strains, Burnet, McCrea and Stone(5) were able to construct a virus "receptor gradient". Thus mumps virus, dose for dose, had less "enzymic activity" than swine influenza virus, and all other strains were intermediate in action.

The original observations that rabbits responded with less fever to a second injection of virus were made with the PR8 and Lee influenza strains and NDV(2). In these studies it was found that tolerance to heterologous virus challenge coincided with the position of the blocking agent in the receptor gradient. It is apparent now that this relationship is purely fortuitous. French(3) has confirmed in essence the resistance of rabbits to repeat injections of virus but he found that the degree of tolerance was dependent on dose and not receptor-destroying activity. Thus animals treated with a sufficiently large amount of mumps, Melbourne A, Lee B or swine influenza viruses were resistant to the fever-producing effects of all of these viruses. Wagner(6) has also reported that the cross tolerance in mice to the neurotoxic action of the WS and Lee influenza viruses was unrelated to the relative receptor-destroying activities of these strains. In addition, treatment of rabbits with the purified receptor-destroying principle (RDE) of *Vibrio comma* filtrates does not modify the febrile response to influenza viruses(3) although crude cholera filtrates possess antipyrogenic(3,7) and antineurotoxic(6) activity.

It would appear, therefore, that a property common to all strains of the influenza-mumps-

NDV group thus far tested is their capacity to produce fever in rabbits. The close relationship of these viruses is further emphasized by their ability to produce tolerance to a second injection of heterologous virus. These phenomena are correlated with hemagglutinating activity(1-3) but do not appear to be related to other biological properties.

Summary. Intravenous injection of mumps virus causes a febrile response in rabbits similar to that produced by influenza or Newcastle disease viruses. Animals are partially or completely resistant to a second injection of mumps virus 24 hours later. Pretreatment with the PR8 strain of influenza A virus also inhibits the febrile response to mumps virus. Mumps virus itself is equally effective in producing tolerance to the PR8 strain.

1. Wagner, R. R., Bennett, I. L., Jr., and LeQuire, V. S., *J. Exp. Med.*, 1949, v90, 321.
2. Bennett, I. L., Jr., Wagner, R. R., and LeQuire, V. S., *J. Exp. Med.*, 1949, v90, 335.
3. French E. L., *Aust. J. Exp. Biol. M. Sc.*, 1952, v30, 479.
4. Salk, J. E., *J. Immunol.*, 1944, v49, 87.
5. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Brit. J. Exp. Path.*, 1946, v27, 228.
6. Wagner, R. R., *Brit. J. Exp. Path.*, 1952, v33, 157.
7. Wagner, R. R., and Bennett, I. L., Jr., *J. Exp. Med.*, 1950, v91, 135.

Received June 19, 1953. P.S.E.B.M., 1953, v83.

Effect of Growth Hormone and Antibiotics upon Nitrogen Mustard Treated Rats.* (20436)

MARVIN L. MITCHELL† AND RENÉ J. GIRERD.
From the Institute of Experimental Medicine and Surgery, University of Montreal, Montreal, Canada.

The remarkable similarity in catabolic patterns resulting from treatment with either total body X-irradiation or systemic nitrogen mustard has been attested by several investigators(1-9). Selye *et al.* have reported upon the anticatabolic effect of somatotrophic hormone (STH) in rats that had received LD_{50} of whole body X-irradiation(10). There have also been gratifying results achieved with the use of antibiotics in animals exposed to ionizing rays(11-15). In contrast to this, the use of antibiotics in acute and chronic nitrogen mustard intoxication has been difficult to evaluate(9,16). It therefore seemed of interest to study the influence of STH and antibiotics, singly and in combination, on the catabolism produced in animals treated with nitrogen mustard.

Material and methods. Seventy male Sherman rats, initial body weight between 60-65 g, were divided into 7 groups of 10 animals per group. Group I served as absolute controls. Group II received penicillin combined with dihydrostreptomycin (Combiotic).‡ Group III received STH.§ Group IV received nitrogen mustard (HN-2) Methyl bis β-Chlorethyl Amine Hydrochloride‖). Group V received HN-2 and Combiotic. Group VI received HN-2 plus STH. Group VII received HN-2, Combiotic and STH.

The animals that were treated with HN-2 received only a single subcutaneous injection of 0.09 mg at the start of the experiment. The HN-2 was prepared immediately prior to administration. One hour later, all the animals were started on their respective treatment with Combiotic, STH, or the combined therapy.

The STH (Armour lot no. R-285-183) was made up freshly every second day with sterile physiological saline, and administered subcutaneously in 2 daily 8 hour intervals, each

---

* This investigation was supported in part by a grant from the Defense Research Board of Canada.

† Public Health Research Fellow of the National Heart Institute.

‡ Combiotic was supplied through the generosity of Pfizer Canada, Ltd.

§ STH was made available through the courtesy of the Armour Laboratories, Chicago, Ill.,

‖ Nitrogen mustard (Mustargen) was kindly provided by Merck and Co., Inc., Rahway, N. J.

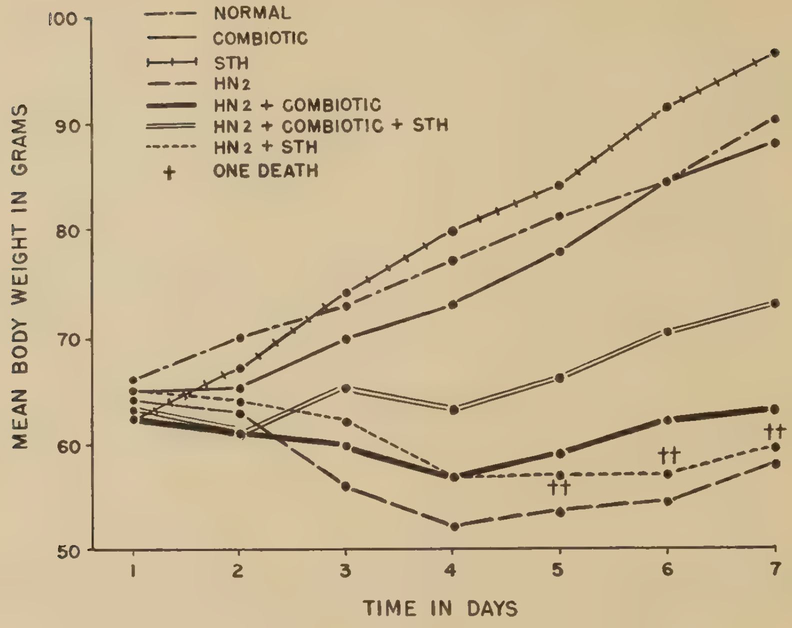


FIG. 1. Differences in mean body wt of all groups.

dose being 1.5 mg. When not in use, the STH solution was stored in the cold room at a temperature of plus 5°C.

The Combiotic consisted of a dry powder containing penicillin G procaine, penicillin G sodium and dihydrostreptomycin. It was diluted daily with sterile physiologic saline so that each 0.2 cc dose delivered 10,000 units of penicillin and 25 mg of dihydrostreptomycin. Injection was once daily via the subcutaneous route.

The diet consisted of Purina Fox Chow with water offered *ad libitum.*

Body weights were recorded daily for a period of 7 days. At the end of that time, all the surviving animals were sacrificed. At autopsy, the liver, spleen, kidneys, adrenals and thymus were taken for weighing and histologic examination.

Results. Examination of Fig. 1 reveals that the groups having received HN-2 (Groups IV-VII) suffered a maximum weight loss between the fourth and fifth days. Therefore, the fourth day served as the point of reference from which differences in mean body weights of the individual groups were calculated (Table I).

In neither of the HN-2 treated groups, receiving Combiotic (Group V) or STH (Group VI), was there any significant degree of protection afforded against the marked loss in body weight. In direct contrast to this, the HN-2 treated group receiving the combined therapy with both STH and Combiotic (Group VII) exhibited an unusually great resistance against the catabolism (Table I).

The only group to suffer any mortality was that receiving HN-2 and being treated with STH (Group VI). Two animals died successively on the fifth, sixth and seventh days (Fig. 1). Autopsy failed to reveal the specific cause of death. Although there was some evidence of scattered infections in the animals that were injected with HN-2, there was none to be seen in the rats dying spontaneously.

In those animals which had received HN-2 alone or combined with other therapy, relative degrees of atrophy of the thymico-lymphatic organs and spleen were observed. The kidney and liver were somewhat smaller than normal, but not significantly so. The adrenal glands were approximately the same size in all groups, regardless of treatment. In general, the organ weight tended to parallel the body weight of the animals.

Discussion. Although our initial concern

TABLE I. Differences in Mean Body Weights and Standard Error on the 1st and 4th Days.

Group	Treatment	Mean body wt and stand. error		P			
		1st day	4th day				
I	Absolute control	65.2 ± 1.4	77.2 ± 2.2	<.01			
II	"Combiotic"*	64.5 ± 1.7	73.4 ± 2.4				
III	STH	63.1 ± 3.2	78.8 ± 3.3				
IV	Nitrogen mustard	63.8 ± 1.9	52.6 ± 1.7	<.01		>.05	>.1
V	Nitrogen mustard + "Combiotic"	63.4 ± 1.5	57.7 ± 1.9		<.05		
VI	Nitrogen mustard + STH	64.9 ± 1.4	57.0 ± .4				
VII	Nitrogen mustard + "Combiotic" + STH	63.3 ± .4	63.3 ± 2.1				

* Combiotic composed of penicillin G procaine, penicillin G sodium and dihydrostreptomycin.

had been the influence of STH and antibiotics on the catabolism, the effect of these drugs on the mortality is of interest. In this, and other unpublished data, there is a strong indication that STH plus HN-2 proved far more fatal than HN-2 by itself. Conversely, Combiotic alone, or in association with STH, appeared to decrease and, in some instances, prevent the lethal action of HN-2. The sensitizing ability of STH is difficult to interpret in terms of its protective property when given to X-irradiated rats(10). Possibly, the deleterious phenomenon could be attributed to the generalized somatic and chemical response elicited by the STH. It has been shown that STH has a marked propensity for provoking protein anabolism(17). Therefore, the increased demand for cellular synthesis, on a previously weakened organism, could further exhaust the remaining resources, thereby lowering resistance and hastening collapse.

One further observation was made in similar experiments in which several groups of HN-2 treated rats were allowed to survive for a 7 week period. In these animals, notwithstanding therapy, a permanent stunting of growth was observed, reminiscent of the immature cortisone treated rats(18).

Reviewing Fig. 1 and Table I, it can be seen that the combination of Combiotic and STH provided very effective protection against the HN-2 effects. The Combiotic alone proved to have some protective quality, while the STH alone was completely without benefit. Since the therapeutic use of HN-2 is limited in part by its toxicity, it would be of interest to determine whether our results have any clinical application.

Summary. A study has been made to determine the influence of STH and antibiotics on HN-2 treated rats. Our results indicate that the combination of STH and antibiotics provided the most highly effective protective action against the HN-2 induced catabolism. STH alone appeared to aggravate the toxic phenomenon, whereas the action of antibiotics alone proved to be of some benefit in preventing the extreme catabolic manifestations of HN-2.

---

1. Gilman, A., and Phillips, F. S., *Science,* 1946, v103, 409.

2. Graef, I., Karnofsky, D. A., Jager, V. B., Krichesky, B., and Smith, H. W., *Am. J. Path.,* 1948, v24, 1.

3. Boyland, E., *Nature,* 1948, v161, 226.

4. Smith, T. R., Jacobson, L. O., Spurr, C. L., Allen, J. G., and Block, M. H., *Science,* 1948, v107, 474.

5. Becker, A. M., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 247.

6. Bukantz, S. C., Dammin, G. J., Wilson, K. S., Johnson, M. C., and Alexander, H. L., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 21.

7. Bennett, I. L., and Cluff, L. E., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 304.

8. Schlang, H. A., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 274.

9. Hammond, C. W., Tompkins, M., and Miller, C. P., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 137.

10. Selye, H., Salgado, E., and Procopio, J., *Acta Endocrinol.,* 1952, v9, 337.

11. Howland, J. W., Furth, F. W., Bennett, L. R., Coulter, M., and McDonnel, G. M., UR-94, The University of Rochester, 1949, 3.

12. Miller, C. P., Hammond, C. W., and Tompkins, M., *Science,* 1950, v3, 719.

13. Howland, J. W., Furth, F. W., and Coulter, M. P., *Fed. Proc.*, 1951, v10, 67.

14. Furth, F. W., Coulter, M. P., and Howland, J. W., *Am. J. Path.*, 1952, v28, 25.

15. Evans, E. I., *Symposium on Treatment of Trauma in the Armed Forces*, March, 1952, 28.

16. Second Report on Institutional Research Grants of the American Cancer Society, September, 1950-August, 1951, 50.

17. Li, C. H., and Evans, H. M., *Recent Progress in Hormone Research*, 1948, v3, 3, Academic Press, Inc., New York.

18. Parmer, L. G., Katonah, F., and Angrist, A. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 215.

Received June 21, 1953. P.S.E.B.M., 1953, v83.

Ineffectiveness of *in vivo* Dialysis in Prolonging Life in X-Irradiated Dogs.* (20437)

PAUL S. LAVIK, JACK R. LEONARDS, G. WARREN BUCKALOO, CHARLES HEISLER, AND HYMER L. FRIEDELL.

From the Atomic Energy Medical Research Project and the Department of Biochemistry, Western Reserve University School of Medicine, Cleveland.

One possible explanation for the lethal effects of ionizing radiation is the accumulation of toxic substances in the body. Experimental evidence both for and against this suggestion has been adequately reviewed by Lawrence, Valentine, and Dowdy(1). Assuming that such a toxic substance did accumulate as a consequence of irradiation and that this substance was readily dialyzable, it might be possible to reduce its concentration to non-toxic levels, by means of an "artificial kidney," and thus decrease or prevent the damaging effects of radiation. The present experimental study was designed to explore this possibility.

Methods and materials. All animals employed in this study were adult mongrel dogs of both sexes ranging in weight from 12.7 to 24.0 kg, and they were fed a regular stock diet of 20% horse meat and 80% commercial dog chow. The "artificial kidney" and the technics employed for the *in vivo* dialysis of the blood have been previously described(2,3). Blood from a femoral artery was pumped at a rate of approximately 200 ml per minute through the dialyzer and back into the animal via the femoral vein. Flowing through the dialyzer in the opposite direction, and separated from the blood by 20,000 sq cm of cellophane membrane, was the dialyzing solution which contained glucose at a concentration of 0.1% and the principal electrolytes at the same concentration as found in the extracellular fluid(2). The dialysis treatment usually lasted for a period of 3-4 hours, during which time approximately 100 liters of dialyzing solution was pumped through the dialyzer. The efficiency of this procedure is shown in Fig. 1 by the rate at which previously injected Na^{24} can be removed from the dog. Sham-

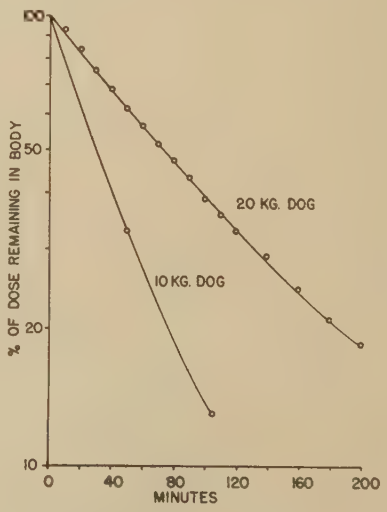


FIG. 1. Effectiveness of "artificial kidney" in removing Na^{24} from dogs.

---

* This work was performed under A. E. C. Contract with Western Reserve University, and partially supported by a grant (to Dr. J. R. Leonards) from the National Heart Institute, of the National Institute of Health, Public Health Service.

dialysis consisted of exactly the same procedure except that no dialyzing solution was put through the dialyzer. The radiation dosages reported in this paper are defined as the number of roentgen units delivered at the top surface of the animal and were measured "in air" at the center of the beam with a Victoreen "r" meter. The x-ray beam in every instance was filtered through 0.5 mm of Cu and 1.0 mm of Al, which corresponds to a HVL of 1.35 mm of Cu. Variations in the radiation procedure were as follows:

Dosage administered		X-Ray machine	
Total r	r/min.	KV	ma.
500	6	220	15
550	6	220	9

Experimental and results. Ten dogs (Group I) were given 550 r whole body x-irradiation but no dialysis treatment. Ten comparable dogs (Group II), which also received 550 r x-irradiation, were treated on the assumption that a toxic factor might be produced during or immediately following x-irradiation. Dialysis was, therefore, begun about 5 minutes before, and was continued for 1½ hours after the irradiation period. The results summarized in Table I show an 80% mortality in both groups at 32 days after irradiation, and the apparent decrease in the average survival time of the treated animals from 17 to 12 days is of questionable significance.

Experimental groups III and IV deal with the effect of the dialysis and sham dialysis procedures respectively when administered 0.5 to 3.0 days after x-irradiation. A total of 20 animals (Group III) were subjected to the dialysis procedure, and of these 10 received 550 r and 10 received 500 r whole-body x-irradiation. To determine whether the irradiated animal might become less able to withstand the dialysis procedure, 13 comparable dogs (Group IV) were sham-dialyzed one-half to 3 days after receiving a whole body x-irradiation dose of either 500 r (8 dogs) or 550 r (5 dogs). Thus, any difference in the survival of the animals in the 2 groups could be attributed to an effect of actually dialyzing the blood. Although every dog in both groups died before the 32nd post-irradiation day, the

TABLE I. Effect of *In Vivo* Dialysis on Survival of X-Irradiated Dogs.

Group	No. of dogs	Time treatment begun	Accumulative deaths during post-irradiation period (in days) 1	2	4	6	8	10	12	14	16	18	20	22	24	26	28	32	% dead at 32 days	Avg survival times (days)
I Control	10	None	0	0	0	0	1	1	2	4	5	5	5	5	7	7	8	8	80	17 ± 6.6*
II Dialyzed	10	Immed. before irrad.	0	1	1	2	4	4	4	4	5	6	7	8	8	8	8	8	80	12 ± 6.6
III Dialyzed	20	.5-3 days after irrad.	2	3	5	7	8	11	14	19	19	20	20	20	20	20	20	20	100	8.9 ± 4.8 (8.8 ± 2.9)†
IV Sham-dialyzed	13	.5-3 days after irrad.	0	0	0	0	4	6	6	10	11	12	12	12	12	12	12	13	100	13 ± 6.4 (10.4 ± 2.8)†

* Stand. dev.

† Includes only animals given 500 r.

dialyzed animals died somewhat earlier than the sham-dialyzed dogs (Table I, groups III and IV). None of the latter survived less than 7 days after irradiation, whereas 7 of the 20 dialyzed dogs died within the same period. The average survival time for the dialyzed animals was only 8.9 ± 4.8 days as compared to 13 ± 6.4 days for the sham-dialyzed dogs. However, if we compare only the results obtained with dogs which were given 500 r, the difference in the survival times for the dialyzed and the sham-dialyzed animals was not so marked (8.8 ± 2.9 vs. 10.4 ± 2.8 days, respectively). Greater significance is attached to these experimental results since the animals were selected with more care to insure that the physical condition of the animals used in the 2 groups was similar at the start of the experiment, and, furthermore, the dialysis and sham-dialysis procedures were both carried out on the same day with the same artificial kidney set-up.

The body weight, rectal temperature, erythrocyte and platelet count, clotting time, and hematocrit readings were comparable in all irradiated dogs regardless of treatment. A comparison of the total leukocyte counts in the irradiated dogs of the different groups showed no important differences after the third day, *i.e.*, a severe leukopenia developed in all dogs regardless of the dialysis treatment. However, a temporary but striking leukocytosis appeared within 24 hours after the animals were dialyzed, and it lasted for about 24-48 hours. As to the mechanism of this leukocytosis, it is not known whether it represents an increased production of cells or simply a release of preformed cells; at any rate, the capacity of the irradiated dogs to respond in this manner disappeared 36 or more hours after irradiation.

Discussion. The success of the *in vivo* dialysis procedure in improving the survival of x-irradiated dogs depends upon the validity of the following assumptions: 1) the lethal effect of radiation is mediated through the action of a toxic substance, 2) the dialysis procedure employed would be effective in reducing the concentration of such a factor(s) to a non-toxic level, and 3) the time for dialysis would be correctly selected and of sufficient duration to be of real therapeutic value. It is evident that the experiments described in this paper provide no evidence as to the presence or absence of toxic factors which are either predominantly intracellular in their distribution or are not readily dialyzable for any reason (*e.g.*, protein binding). However, some conclusions might be drawn as to the role of possible toxic factors that are readily dialyzable and which are located primarily in the extracellular fluids. If the results obtained with Na^{24} typify the effectiveness of the dialysis procedure in removing such factors from the body, it seems doubtful that their presence at the time of irradiation, or immediately after irradiation, was responsible for the subsequent death of the irradiated animals. Furthermore, if there was a gradual accumulation of such substances until toxic levels were reached, a temporary reversal of this process by the dialysis treatment at 1-3 days after irradiation might have been expected to decrease the mortality rate. This was not observed. The effect of more prolonged treatments administered later in the post-irradiation period was not determined.

Summary and conclusions. Dogs given 500-550 r whole body x-irradiation were subjected to an *in vivo* dialysis of their blood during the early post-irradiation period. No improvement in the survival of these animals was noted. When applied 0.5-3.0 days after irradiation, the dialysis procedure caused a slight decrease in the average survival time, as compared to sham-dialyzed controls. The results tend to minimize the importance of readily dialyzable "toxins" in the lethal action of x-irradiation.

---

1. Lawrence, J. S., Valentine, W. N., and Dowdy, A. H., *Blood,* 1950, v3, 593.

2. Skeggs, L. R., Leonards, J. R., and Heisler, C. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 539.

3. Leonards, J. R., and Heisler, C. R., *Am. J. Physiol.,* 1951, v167, 553.

Received June 22, 1953. P.S.E.B.M., 1953, v83.

Suspended Cell Tissue Cultures for Study of Virus Growth Kinetics.* (20438)

D. CARLETON GAJDUSEK. (Introduced by J. F. Enders)

From the Research Division of Infectious Diseases, Children's Medical Center, Boston, Mass.

The embryonated hen's egg, the deembryonated egg, and, more recently, tissue cultures using the chick embryo chorioallantoic membrane have been widely used in the study of the growth of influenza virus(1-13). The present investigation was undertaken to appraise the suspended cell tissue culture for the quantitative determination of the multiplication of this agent. Attention has been focused on early virus production as measured by titration of infectivity in tissue culture flasks.

Materials and methods. Virus. The PR8 strain of influenza A virus propagated in chick chorioallantoic membrane tissue culture has been used in all experiments. This agent was received from Dr. Frank Horsfall 15 years ago and has been maintained since then by allantoic passage in the chick embryo. The stock virus consisted of the fluid removed from cultures inoculated 36 to 48 hours previously with 0.1 ml of infected allantoic fluid diluted 10^{-3}. It was stored at —70°C in glass-sealed ampules.

"Titration" flasks. For titration of viral infectivity at various intervals tissue culture flasks such as those used by Enders and his coworkers(14,15) were prepared with scissor-minced chorioallantoic membrane tissue from 9-11 day chick embryos. Three ml of a mixture consisting of 3 parts Hanks' balanced salt solution and one part Simms' ox serum ultrafiltrate was added to each 25 ml Erlenmeyer flask. The medium also contained 50 units of penicillin and 50 μg of streptomycin per ml. A 50% suspension of the minced tissue was prepared by centrifuging the fragments at 1000 rpm for 5 minutes and adding the required volume of Hanks-Simms' solution. An equal number of drops (2 or 3) of the well agitated suspension was delivered into each flask from a large bore capillary pipette avoiding the first and the last few drops in the pipette. Inspection of the flasks revealed that tissue could be distributed in this way with less than a 2-fold variation in quantity (counts of fragments) from flask to flask. The flasks were stoppered tightly and incubated at 35°C.

"Reaction" flasks. To avoid significant changes in the volume of the culture medium that would result from the repeated removal of aliquots for titration from cultures containing small quantities of fluid a larger system was employed for the study of virus multiplication. To Erlenmeyer flasks of 250 ml capacity 21 ml of Hanks' balanced salt solution (without serum ultrafiltrate) containing 50 units of penicillin and 50 μg of streptomycin per ml were added. The source of tissue was chorioallantoic membrane from 9-day embryos. Pooled membranes were minced, and the tissue was washed 5 times with Hanks' solution. After each washing the tissue was sedimented by centrifugation at low speed. Seven times as much tissue was employed as in the cultures used for titration. After addition of the virus the flasks were stoppered tightly and incubated at 35°C.

Experimental results. I. *Parallel titrations in tissue cultures and chick embryos.* On the same day parallel titrations of viral infectivity were carried out in tissue culture and in embryonated eggs using a series of dilutions based on a factor of $10^{0.5}$. In each titration the same volume of each dilution was added within a few minutes to groups of flasks and inoculated into the allantoic cavity of embryonated eggs. The endpoint was determined by testing the freshly harvested egg fluid from each egg or tissue culture for the presence of hemagglutinin. The results of 2 parallel titrations are summarized in Tables I and II. Two different preparations of virus were titrated.

II. *Growth of virus in tissue culture following introduction of small inocula.* The increase in infectivity of the supernatant fluid

---

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

TABLE I. Procedures and Results of Simultaneous Titrations of Influenza PR8 Virus in Tissue Cultures and Eggs.

	I	II
Age of chorioallantoic membrane used in flasks	10 days	9 days
Incubation period before inoculation of eggs	12 "	11 "
Inoculum vol used for eggs and for flasks	0.1 ml	0.2 ml
Period inoculated eggs incubated before harvesting	2 days	2 days
Period inoculated flasks incubated before harvesting	6 "	6 "
Titer* in flasks	$10^{-3.4}$	$10^{-4.8}$
Titer* in eggs	$10^{-4.4}$	$10^{-5.8}$

* All infectivity endpoints were calculated by the method of Karber, G., *Arch f. Exp. Path. u. Pharmakol.*, 1931, v162, 480.

TABLE II. Comparison of Tissue Culture and Egg Titration of Influenza PR8 Virus.

	I		II	
Dilution	Eggs	T.C.	Eggs	T.C.
$10^{-2.5}$	6/6	6/6	—	—
$10^{-3.0}$	5/6	5/6	7/7	5/5
$10^{-3.5}$	5/6	3/6	7/7	5/5
$10^{-4.0}$	4/6	0/6	7/8	5/5
$10^{-4.5}$	3/6	0/6	6/8	3/5
$10^{-5.0}$	2/6	0/6	5/8	1/5
$10^{-5.5}$	—	—	2/8	0/5
$10^{-6.0}$	—	—	2/8	1/5
$10^{-6.5}$	—	—	1/7	—
Titer	$10^{-4.4}$	$10^{-3.4}$	$10^{-5.8}$	$10^{-4.8}$

from tissue culture flasks has been studied during the first 72 hours after inoculation. Influenza virus was mixed with the tissue culture medium at 35°C and the mixture was immediately added to the washed and minced tissue in the reaction flask. A sufficient quantity of virus was added to yield a final concentration of 10 ID_{50} (for tissue culture) per ml of the medium. At frequent intervals 0.5 ml of the supernatant fluid from the reaction flask was withdrawn and promptly titrated in tissue cultures. The samples were centrifuged immediately in a Sorvall centrifuge at 13,000 rpm for 10 minutes to remove tissue elements. Two-fold serial dilutions of the supernatant fluids were prepared with isotonic phosphate buffer pH 7.1 and aliquots inoculated into titration flasks. Usually 3 cultures, although occasionally only 2, were used for each dilution; in a few instances 5 or more were employed. After 5 to 7 days the supernatant fluids from each titration flask were tested for hemagglutinin. In every case titration flasks were inoculated within an hour after a sample was withdrawn from the reaction flask. For infectivity titrations of fluids a series of dilutions based on a factor of 2 were employed.

After several preliminary experiments in which the approximate ranges of infectivity were determined at 2-hour intervals following inoculation, large-scale experiments were carried out in which the variation in the titer in one reaction flask was determined during a period of 36 to 72 hours. The results of 2 such experiments are presented in Fig. 1 and 2. The titers are expressed as ID_{50} per 0.1 ml (for tissue culture) of supernatant fluid from the reaction flask. The inoculum for the reaction flask consisted one ID_{50} per 0.1 ml of medium. This was verified by titration of a sampled aliquot in tissue culture flasks immediately after addition of the medium containing virus to the tissue in the reaction flask. The vertical lines drawn through the points represent the variation in titer that might be expected to occur in different determinations of the infectivity on a single specimen of virus. In repeated experiments the titer of a given fluid did not vary by more than a factor of 2.

Discussion. The results of infectivity titrations of influenza PR8 virus in tissue culture flasks and embryonated eggs have been compared. It appears that the egg is a more sensitive indicator of the presence of virus than the tissue culture as employed in these experiments. Infectivity endpoints in cultures are sharp but the endpoints in eggs are higher by a factor of $10^{0.5}$ or $10^{1.0}$. The possibility of increasing the sensitivity of tissue culture infectivity titrations by agitation or rocking or of adopting another type of culture such as the roller tube has not been tested. Fluids were tested for hemagglutinin at any time from the fifth to the ninth day after inoculation of the flasks. Hemagglutinin had not emerged after the fifth day, and no positive flasks became negative for hemagglutinin on standing until the ninth day. Dilution steps based on a factor of 2 have been successfully employed using 3 or more flask replicates for each dilution. For relative estimates of virus

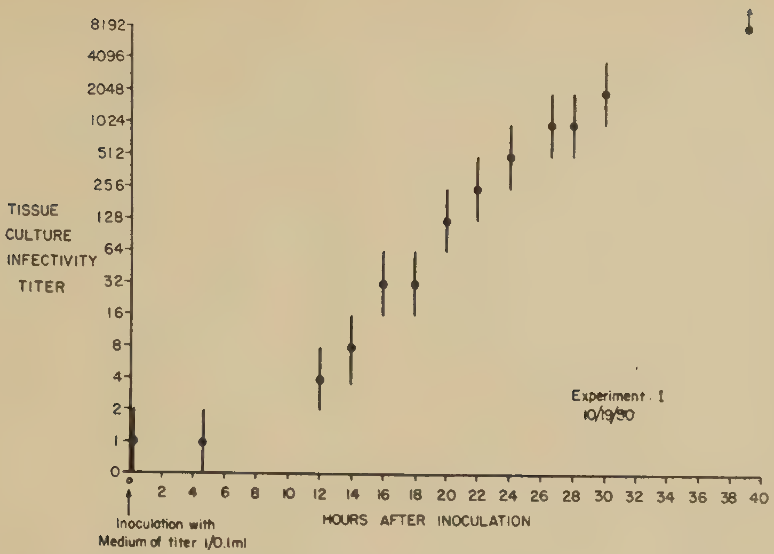


FIG. 1. Growth of influenza PR8 virus in chick embryo chorioallantoic membrane suspended cell tissue culture (large flask).

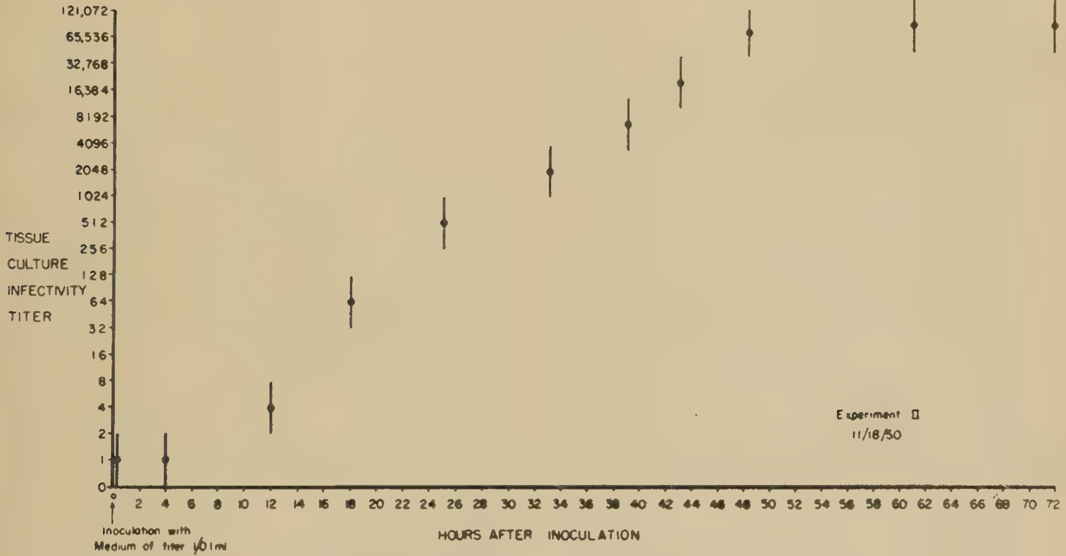


FIG. 2. Growth of influenza PR8 virus in chick embryo chorioallantoic membrane suspended cell tissue culture (large flask).

infectivity tissue culture flasks provide a uniform testing system, simple to prepare in large numbers and easy to manipulate. Six to 8 flasks may be prepared from a chorioallantoic membrane derived from one 10-day embryo.

The growth curves in Fig. 1 and 2 illustrate the reproducibility of results that may be obtained with the technics here described. The 2 experiments were conducted one month apart employing the same infecting dose of virus. Several other similar experiments gave equally consistent results. Since these early growth curves are consistently reproducible it may be concluded that the procedure may be employed as a means of determining the effect of variation in the physical conditions or composition of the culture medium on viral multiplication. Even relatively small changes in the shape of the curve would appear to be significant.

Several curves reflecting the decay of virus infectivity at 35°C and at various pH ranges in Hanks' balanced salt solution with the addition of antibiotics but in the absence of tissue have been determined for different initial concentrations of virus. After 4 hours there was no measurable loss of virus infectivity, but after 24 hours this had declined by a factor of $10^{0.7}$ from an initial titer of $10^{-4.3}$. Although it may be objected that such decay curves may not represent the loss of infectivity occurring in reaction flasks *in the presence of living tissue,* the correction of the growth curves of Fig. 1 and 2 for decay as determined in the absence of tissue might be justified.

For accurate growth kinetic studies of mammalian viruses similar to those available to workers with bacteriophage the suspended fragment tissue culture is far from the ideal, *i.e.,* cultures of uniformly suspended pure strain cells which may be accurately quantitated. Progress toward this ideal has been made by Evans and her coworkers(16,17) who have developed pure tissue culture lines from single cells and succeeded in producing quantitatively uniform replicate cultures by counting the viable cell nuclei in cell suspensions of their tissue inocula. But for the present, the simple suspended fragment technic here employed provides a technically uncomplicated and fairly accurate means for the quantitative evaluation of the multiplication of certain viruses in tissue culture.

The author wishes to thank Dr. John F. Enders, Dr. Frederick C. Robbins and Dr. Thomas H. Weller for their helpful suggestions and criticisms during this study.

---

1. Henle, W., *J. Exp. Med.,* 1949, v90, 1.

2. ———, *J. Exp. Med.,* 1949, v90, 13.

3. Henle, W. and Henle, G., *J. Exp. Med.,* 1949, v90, 23.

4. von Magnus, P., *Acta Pathol. et Microbiol. Scand.,* 1951, v28, 250.

5. ———, *Acta Pathol. et Microbiol. Scand.,* 1951, v28, 278.

6. ———, *Acta Pathol. et Microbiol. Scand.,* 1951, v29, 157.

7. ———, *Acta Pathol. et Microbiol. Scand.,* 1951, v30, 311.

8. Bernkopf, H., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 680.

9. ———, *J. Immunol.,* 1950, v65, 571.

10. Rasmussen, A. F., and Stokes, J., *J. Immunol.,* 1951, v66, 237.

11. Fulton, F., and Armitage, P., *J. Hygiene,* Cambridge, 1951, v49, 247.

12. Fulton, F., *J. Hygiene,* Cambridge, 1952, v50, 265.

13. Daniels, J. B., Monroe, E. E., and Perry, M. E., *J. Immunol.,* 1952, v69, 321.

14. Weller, T. H., and Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 124.

15. Weller, T. H., Enders, J. F., Robbins, F. C., and Stoddard, M. B., *J. Immunol.,* 1952, v69, 645.

16. Evans, V. J., In *Methods of Medical Research,* 1951, v41, The Year Book Publishers, Inc., Chicago. (Maurice B. Visscher, ed.).

17. Evans, V. J., Earle, W., Sanford, K. K., Shannon, J. E., and Waltz, H. K., *J. Nat. Cancer Inst.,* 1951, v11, 907.

Received June 22, 1953. P.S.E.B.M., 1953, v83.

Effect of Zinc on Cytochrome Oxidase Activity. (20439)

G. D. DUNCAN, LOUISE F. GRAY AND LOUISE J. DANIEL. (Introduced by L. C. Norris)

From the U. S. Plant, Soil and Nutrition Laboratory, Bureau of Plant Industry, Soils and Agricultural Engineering, A.R.A. and the Department of Biochemistry and Nutrition, Cornell University, Ithaca, N. Y.

Excess dietary zinc causes a hypochromic, microcytic anemia in rats(1-3). The anemia is wholly or partially prevented by feeding additional copper(2,3). The need for copper in hemoglobin formation was discovered by Hart, Steenbock, Waddell and Elvehjem(4). Cohen and Elvehjem(5) and Schultze(6,7) reported that cytochrome oxidase activity was decreased in livers, hearts and bone marrow of rats fed a copper-deficient diet. Schultze and Kuiken(8) found that liver catalase activity was also decreased in copper deficiency. Thus, it is evident that copper is concerned with the formation of iron-porphyrin compounds.

Since zinc has been shown to induce a copper deficiency state in rats, this investigation was undertaken to determine the effect of dietary zinc on the activity of cytochrome oxidase in heart tissue.

Experimental. Animal studies. Rats originating from the Sprague-Dawley strain were started on experimental diets at the age of 21 to 25 days. Six rats (3 males and 3 females) were placed on each of 7 treatments. The basal diet was whole milk powder* to which mineral supplements of ferrous sulfate, cupric sulfate and manganous sulfate had been added. The rats on the basal diet received approximately 0.06 mg of copper, 0.38 mg of iron and 1 mg of manganese per day as a result of this supplementation. The following diets were fed *ad libitum* for a period of 5 weeks: 1) Basal, 2) Basal + 1.0% zinc, 3) Basal + 1.5% zinc, 4) Basal + 0.03% copper, 5) Basal + 1.0% zinc + 0.03% copper, 6) Basal + 1.0% zinc + 1 mg % folic acid, 7) Stock diet.† A-D Percomorph Liver Oil was administered weekly to each rat by dropper. Growth and hemoglobin were determined at the end of the experimental period. The animals were then sacrificed and cytochrome oxidase activity of the heart tissue was measured.

Enzyme studies. The cytochrome oxidase activity was determined as follows. The animal was decapitated and the heart removed at once. It was divided longitudinally and one half of it placed in 5.0 ml of ice cold 0.1 M potassium phosphate buffer, pH 7.2. The tissue was homogenized for 2 minutes in a Potter-Elvehjem glass homogenizer surrounded by an ice-water bath, and the sample was then diluted to the desired concentration with 0.1 M phosphate buffer. The diluted homogenate was allowed to stand exactly 5 minutes in an ice-water bath to permit large particles to settle out before pipetting into the reaction vessels. The enzyme activity was determined by the manometric method of Slater(9) with some minor modifications of reagent concentrations. Four concentrations of cytochrome *c* (Sigma Chemical Co.) were used at each of two levels of enzyme for a complete determination. The ferrocytochrome *c* was standardized spectrophotometrically using a molecular extinction coefficient at 550 mμ of 27,840(10). The test system consisted of 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.2), 0.3 ml of 4 x 10^{-3} M aluminum chloride, 0.3 ml of 0.114 M sodium ascorbate, 1.0, 0.8, 0.7 or 0.6 ml of 1.94 x 10^{-4} M cytochrome *c*, and 0.2 or 0.3 ml of enzyme suspension containing approximately 1 mg of organic matter per ml. The volume of the reaction mixture was made to 3.0 ml by additions of heavy-metal-free water. The enzyme suspension was added last. Sodium hydroxide was found to be unnecessary in the center well of the Warburg flask. After a 10-minute equilibration period, oxygen consumption was measured at 10-minute intervals for a 40-minute period in a Warburg apparatus at 37°, with air as the gas phase. The activity was expressed as oxygen uptake in microliters

---

* Klim, The Borden Co., New York City.

† Big Red G.L.F. Dog Food Pellets, Cooperative G.L.F. Marketing Service, Inc., Canandaigua, N. Y.

TABLE I. Effect of Treatment on Growth, Hemoglobin Level and Cytochrome Oxidase Activity of Rats.*

Treatment	Wt change, g	Hemoglobin, g/100 ml	Cytochrome oxidase activity, Qo_2†
1. 0‡	149	14.1	1699
2. 1.0% zinc	101	5.5	500
3. 1.5% "	80	4.5	282
4. 0.03% Cu	146	13.8	1774
5. 1.0% zinc + 0.03% Cu	113	9.7	2304
6. 1.0% zinc + 1 mg % folic acid	111	5.0	391
7. Stock diet	161	14.2	1885

* Duration of animal experiment was 5 wk. All values are the mean for 6 animals, 3 males and 3 females.

† $Qo_2 = \mu$l of oxygen uptake/hr/mg of organic matter calculated to infinite concentration of cytochrome *c*. The tissue used was heart.

‡ Mineralized whole milk powder.

(μl) per hour per mg of organic matter. The *organic matter* was determined by the method of Johnson(11), using a 0.1% solution of sucrose as the standard. Duplicate 0.4 ml aliquots of the suspension were taken at the time of pipetting the homogenate into the Warburg vessels, and placed in pyrex test tubes. The color developed was read in a filter colorimeter using Corning filters 3389, 3389, 5113 (½ std. thick) with a transmission peak at 440 mμ with a spread of approximately 75 mμ at the 1% level. The Qo_2 (organic matter) was determined at each level of cytochrome *c*. The reciprocals of the four Qo_2 values were plotted against the reciprocals of the corresponding cytochrome *c* concentrations on rectangular coordinate paper and extrapolated to zero [infinite cytochrome concentration] (9).

Results and discussion. The adverse effect of excess dietary zinc on growth and hemoglobin formation in the young rat has been confirmed in this study. These results are presented in Table I. A marked growth retardation was observed at both the 1.0 and 1.5% levels of zinc ($P < 0.01$). The addition of copper to the diet containing 1.0% zinc improved growth significantly ($P < 0.05$), although this improved growth was still considerably below that obtained for the animals on the basal diet. Since Albert(12) has shown folic acid to be a good chelating agent for heavy metals, this compound was included in one of the treatments to learn if it would reduce the toxic effect of zinc. The results presented indicate that it was without effect.

A level of 1.0% zinc in the diet resulted in a reduction in hemoglobin concentration of about 40% of normal ($P < 0.01$) and the addition of 1.5 zinc caused a further significant decrease ($P < 0.05$). The addition of 0.03% copper to the diet containing 1.0% zinc was effective in partially preventing the anemia ($P < 0.01$). Folic acid had no effect on the hemoglobin level, as might be expected in a microcytic anemia.

The effect of excess dietary zinc on cytochrome oxidase activity is presented in Table I. The results show the enzyme activity was decidedly lower in the presence of excess dietary zinc ($P < 0.01$). When copper alone was added to the basal diet, no change in enzyme activity occurred; however, the addition of copper to the 1.0% zinc diet actually caused an increase in cytochrome oxidase activity above that of the basal animals ($P < 0.01$). The following is a possible explanation of this increase. An anemic animal with a low concentration of hemoglobin must circulate the blood more frequently in an attempt to supply the tissues with the oxygen needed for metabolism. This necessitates a marked increase in heart action. The higher-than-normal enzyme activity may have been the result of the increased need on the part of the heart tissue itself for those enzymes involved in energy production. In this group of rats (copper and zinc) a moderate anemia existed, although the copper supply was adequate for high cytochrome oxidase activity. It is evident from these results that the synthesis of cytochrome oxidase takes precedence over that of hemoglobin. This was also an earlier observation of Schultze(7).

Folic acid did not alter the effect of zinc on cytochrome oxidase activity, and hence these values serve as additional data confirming the deleterious effect of zinc on the activity of this enzyme.

The cytochrome oxidase activity observed in the rats on the stock diet was greater than that of the rats on the basal diet. It is improbable that this difference was due to a higher copper content in the stock diet, since the enzyme activity was not changed when 0.03% copper was added to the basal diet.

It was believed important to eliminate the possibility that the presence of ionic zinc in the homogenates was the cause for the low cytochrome oxidase activity. The concentration of zinc in the heart tissue of the animals on the 1.0% zinc diet was determined by the method of Parks, *et al.*(13). This level of zinc, 80 μg per g of heart tissue (dry weight), was added *in vitro* to heart homogenates of normal rats. No reduction in cytochrome oxidase activity was observed, indicating that the effect of zinc was not occurring in this manner. It is of interest to note that the copper content (13) of the hearts of animals receiving 1.0% zinc was 40% of the normal value. [Normal, 27 μg per g; 1.0% zinc, 10 μg per g (dry weight).]

The coefficient of correlation was determined for the relation between the hemoglobin values and cytochrome oxidase activities. There was a highly significant correlation between these sets of values, $r = 0.79$.

The exact nature of the action of zinc on hemoglobin and cytochrome oxidase formation is not known, although sufficient evidence exists to show that excess dietary zinc induces a copper deficiency state. Since many metals form chelates with protoporphyrin, it may be that zinc is competing with copper in some manner in the formation of iron porphyrins.

Addendum. While this paper was in preparation an abstract was published in the *Fed. Proc., 12,* 283 (1953), describing similar studies by Van Reen and Pearson. Their findings, although on rat liver instead of heart tissue, are in agreement with the results of the studies presented here.

Summary. 1. Excess dietary zinc resulted in lowered growth, hemoglobin concentration and cytochrome oxidase activity in the rat. 2. The addition of copper to the zinc diet increased growth and hemoglobin, but to suboptimum levels, whereas the cytochrome oxidase activity was raised to a greater than normal level. 3. A highly significant correlation was found between hemoglobin level and cytochrome oxidase activity. 4. The inhibitory effect of zinc was not due to the presence in the homogenate of free zinc ions, since the *in vitro* addition of inorganic zinc to normal rat hearts in amounts equivalent to that found in the zinc-toxic hearts had no effect on the activity of cytochrome oxidase.

---

1. Sutton, W. R., and Nelson, V. E., PROC. SOC. EXP. BIOL. AND MED., 1937, v36, 211.
2. Smith, S. E., and Larson, E. J., *J. Biol. Chem.*, 1946, v163, 29.
3. Gray, L. F., and Ellis, G. H., *J. Nutr.*, 1950, v40, 441.
4. Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., *J. Biol. Chem.*, 1928, v77, 797.
5. Cohen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1934, v107, 97.
6. Schultze, M. O., *J. Biol. Chem.*, 1939, v129, 729.
7. ———, *J. Biol. Chem.*, 1941, v138, 219.
8. Schultze, M. O., and Kuiken, K. A., *J. Biol. Chem.*, 1941, v137, 727.
9. Slater, E. C., *Biochem. J.*, 1949, v44, 305.
10. Tint, H., and Reiss, W., *J. Biol. Chem.*, 1950, v182, 397.
11. Johnson, M. J., *J. Biol. Chem.*, 1949, v181, 707.
12. Albert, A., *Biochem. J.*, 1950, v47, ix.
13. Parks, R. Q., Hood, S. L., Hurwitz, C., and Ellis, G. H., *Ind. Eng. Chem.*, 1943, v15, 527.

Received June 22, 1953. P.S.E.B.M., 1953, v83.

Isolation of a Potent Sodium-Retaining Substance from Adrenal Venous Blood of the Dog.* (20440)

Gordon L. Farrell and John B. Richards.† (Introduced by George Sayers)

From the Department of Physiology, Western Reserve University School of Medicine, Cleveland, O.

A potent sodium-retaining substance has been isolated during the course of a study which has been designed to determine the chemical and biological characteristics of steroids in adrenal venous blood of the dog. The substance is many times more active than desoxycorticosterone acetate (DOCA) in producing sodium retention in the adrenalectomized rat. The work confirms the recent report of Simpson *et al.*(1) who isolated a sodium-retaining factor from adrenal venous blood of a dog and from a perfusate of a monkey adrenal.

Methods. Mongrel male dogs were anesthetized with sodium pentobarbital. Adrenal venous blood was collected from a cannula placed in the left lumboadrenal vein. The adrenal vein was ligated between the adrenal gland and the inferior vena cava. Blood pressure was recorded continuously and pooled dog blood was infused at a rate sufficient to maintain the blood pressure at or above 100 mm mercury during the collection period. A liter of adrenal venous blood was collected over 3 to 4 hours. Two liters of arterial blood were collected by intermittent bleeding from the femoral artery during the same period. One liter of this arterial blood was collected in a vessel containing 1500 μg of 17-hydroxycorticosterone and 500 μg of corticosterone for recovery studies. The other liter of arterial blood was analyzed in order to obtain an estimate of the concentration and the types of steroids entering the adrenal. V-A difference represents the contribution of the adrenal to steroids in adrenal venous blood. Blood was diluted with an equal volume of water and extracted twice with a volume of chloroform equal to that of the blood-water mixture. The chloroform extracts were evaporated and the residue partitioned between hexane and 70% ethanol. The 70% ethanol fraction was chromatographed in a propylene glycol-toluene system according to the method of Burton *et al.*(2). The design of chromatography was as follows: 1) the entire extract was first chromatographed for 96 hours on a paper strip impregnated with half-strength propylene glycol;[‡] 2) the overflow from 1) was chromatographed for 24 hours on paper impregnated with full-strength propylene glycol; 3) the overflow from 2) was chromatographed for 4 to 6 hours on paper impregnated with full strength propylene glycol. The more polar components of 1) were rechromatographed for 240 hours. Steroid mixtures on certain areas of the paper were chromatographed several times to effect satisfactory resolution. Steroids were located on the chromatograms by 1) viewing under ultraviolet light, 2) ammoniacal silver nitrate staining of small strips cut from the main chromatogram, and 3) sulfuric acid reaction on small strips cut from the main chromatogram. Finally, each chromatogram was cut at right angles to the length of the strip into 1 or ½ inch pieces. Steroids were eluted from individual cuts with methanol. The methanol solutions were quantitatively analyzed by 1) absorption in the ultraviolet, 2) reaction with phenylhydrazine and 3) reaction with sulfuric acid. The bioassay method employed for determining alterations in urinary electrolyte excretion was adapted from the procedure described by Marcus *et al.*(3). Three modifications of this method were employed throughout the present study; 1) test rats were not

---

* This investigation was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service and by a research grant from The Upjohn Co.

† Present address, Fourth Medical Service, Boston City Hospital, Boston, Mass.

[‡] Paper is impregnated with a propylene glycol-methanol (1:1) mixture and the methanol allowed to evaporate. The highly polar steroids are more effectively resolved under these conditions in which the quantity of propylene glycol per unit weight of paper is reduced.

subjected to a sodium chloride load during the assay period, 2) urine was collected from single rats instead of rat pairs, and 3) the collection of urine was extended to 6 hours.

For each individual assay, 30 male Holtzman rats weighing 150 to 200 g were bilaterally adrenalectomized and given 0.9% sodium chloride solution to drink. On the third day following adrenalectomy, tap water was substituted for the 0.9% sodium chloride solution. On the morning of the fourth day, food and water were removed from the cages, and the rats injected 3 to 4.5 hours later. All substances tested were dissolved in olive oil and injected subcutaneously. Each rat was placed in a one-liter beaker containing a wire mesh screen which separated urine and feces. Bladder emptying was induced prior to injection of test material and at the termination of the collection period by exposing each rat to ether. Following the collection period, the feces were removed from the wire screen, the beaker was washed with a 0.5% lithium nitrate solution and the washings were diluted to 100 ml. Urinary sodium and potassium concentrations were determined with a Perkin-Elmer flame photometer (model 52A).

Results. A. Components of adrenal venous blood. Studies were carried out on 2 male dogs weighing 18 and 15 kg. In each experiment, the following substances were present in higher concentrations in adrenal venous blood than in the arterial blood: 1) 17-hydroxycorticosterone, concentration, 259 and 294 μg/100 ml; 2) Substance 8, concentration, 4.9 and 10.4 μg/100 ml; 3) 11-desoxy-17-hydroxycorticosterone, concentration, 39 and 46 μg/100 ml; and 4) corticosterone, concentration, 116 and 120 μg/100 ml. Substance 8 chromatographed in the same region as cortisone, absorbed at 240 mμ, gave a 400 mμ maximum with phenylhydrazine under conditions employed in this laboratory but not under conditions described by Porter and Silber(4) and gave a tan-colored solution in sulfuric acid with maxima at 285 and 390 mμ. It is noteworthy that in both experiments, 11-desoxy-17-hydroxycorticosterone was secreted by the adrenal; this steroid has not been found to be secreted by the adrenal of all dogs(5). A more complete report of these analyses, which are a part of a long-range study on the nature of the steroid secretion pattern of the dog adrenal cortex, is in preparation(5). The arterial blood to which 17-hydroxycorticosterone and corticosterone had been added contained the same substances in the same concentrations as arterial blood with the exception of the added steroids, which were recovered in approximately 90 and 70% yields, respectively. This experiment indicated that none of the substances found in adrenal venous blood was derived from the major steroids by degradation during extraction and chromatography.

B. Biological activity of Substance 8. The activity of Substance 8 in inducing alterations in urinary sodium and potassium excretion was compared with that of desoxycorticosterone acetate. The results are shown in Table I. From the data presented it is evident that Substance 8 exerted a potent sodium-retaining effect. Maximal sodium retention was induced with 2.0 μg of Substance 8 per rat and with 50 μg of DOCA per rat. 0.7 μg of Substance 8 caused significant sodium retention; however, the sodium excretion that occurred in rats given 0.2 μg of Substance 8 was not significantly different from that of the control untreated animals ($p = 0.4$).

The area of the chromatogram of arterial blood in which Substance 8 would have been expected to appear contained no appreciable quantity of this material as estimated by chemical tests. Nevertheless, the eluate of this area of the chromatogram was tested for sodium-retaining activity in a dose equivalent to 73 ml of arterial blood per rat. The results in Table I show that this region of the chromatogram of arterial blood had no detectable sodium-retaining activity in the amounts of blood employed; the equivalent of 10 to 20 ml of adrenal venous blood induced significant sodium retention.

Ten and 50 μg of DOCA caused significant ($p < 0.01$) increases in potassium excretion in adrenalectomized rats (Table I). It is evident that as the dose of the hormone was increased the urinary excretion of this electrolyte increased. No such graded response in potassium excretion was observed when various doses of Substance 8 of adrenal venous

TABLE I. Urinary Sodium and Potassium Excretion with Various Doses of Substance 8 from Adrenal Venous Blood of Dogs and with Various Doses of DOCA.

Substance tested	Dose/rat	No. rats	Sodium excretion, mg/6 hr	No. rats	Potassium excretion, mg/6 hr
Olive oil (controls)	1 ml	16	5.53 ± .39*	18	7.76 ± .44
DOCA	50 μg	16	2.49 ± .20	15	10.71 ± .33
	10 ”	11	3.45 ± .21	10	9.51 ± .43
	5 ”	5	5.92 ± .48	5	8.79 ± .47
Substance 8 from adrenal venous blood†	3.5 μg	5	2.50 ± .57	5	9.66 ± .77
	2.0 ”	5	2.33 ± .10	5	9.11 ± .61
	1.4 ”	6	2.71 ± .34	6	9.80 ±1.10
	.7 ”	5	3.08 ± .27	4	11.12 ± .62
	.2 ”	6	4.91 ± .61	6	9.26 ± .47
Substance 8 region of arterial blood	73 ml blood	5	6.43 ± .70	5	7.60 ± .66

* Stand. error.

† Quantity estimated by reaction with phenylhydrazine.

blood was injected into rats. Furthermore, the largest dose of Substance 8 used (3.5 μg) produced only a suggestively significant increase in potassium excretion over the quantities excreted by untreated animals ($p = .05$). The relatively high variation in potassium excretion of the assay animals treated with Substance 8 precludes the drawing of final conclusions on the effect of this material on potassium excretion.

Discussion. The findings in this study are in essential agreement with those of Simpson *et al.*(1), who found a substance of high electrolyte activity in dog and monkey adrenal venous blood. The substance described by Simpson *et al.* chromatographed in the same region as cortisone but was not further characterized. Substance 8, the sodium-retaining steroid described in this communication, chromatographed in the same area as cortisone. It appeared to be homogeneous and had the following characteristics: absorbed at 240 mμ in the ultraviolet, reduced ammoniacal $AgNO_3$, reacted with phenylhydrazine to yield a 400 mμ maximum under the conditions of the test as applied in this laboratory(5) and reacted with sulfuric acid to give a tan solution with maxima at 285 and 390 mμ (*cf.* cortisone maxima 280, 340 and 410 mμ). Substance 8 did not give a blue color with iodine reagent (cortisone reacts to give a blue color). The last two findings distinguish the substance from cortisone. It is not possible at this time to state unequivocally that Substance 8 is pure; the chemical characteristics presented may not be those of the sodium-retaining factor but those of a contaminant. If the sodium-retaining steroid is associated with one or more substances, its potency is even greater than that described for Substance 8 which is approximately 25 times as active as DOCA.

The data presented in this report suggest that the sodium-retaining substance from adrenal venous blood has less influence upon potassium excretion than DOCA when the potassium effect is expressed in relation to the sodium effect. Experiments are in progress to determine the influence of this substance on potassium excretion over a wider dose range than employed in the present work. Simpson *et al.*(1) have expressed the activity of their sodium-retaining factor in terms of a ratio of Na^{24} to K^{42}; they have not presented individual figures for Na^{24} and for K^{42}. The results presented in this report were based on total sodium and total potassium excretions and definitely establish the fact that Substance 8 induces sodium retention in the adrenalectomized rat.

Summary. A substance has been isolated from the adrenal venous blood of dogs which is approximately 25 times as potent as DOCA in producing sodium retention in adrenalectomized rats. It has not been established with certainty that this substance is pure. The material is slightly less polar than cortisone, exhibits an absorption maximum at 240 mμ, does not react with the phenylhydrazine reagent described by Porter and Silber, and does react with a phenylhydrazine reagent developed in this laboratory. The substance shows absorption maxima at 285 and 390 mμ when mixed with concentrated sulfuric acid.

---

1. Simpson, S. A., Tait, J. F., and Bush, I. E., *The Lancet*, 1952, v2, 226.

2. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.*, 1951, v188, 763.

3. Marcus, F., Romanoff, L. P., and Pincus, G., *Endocrinology*, 1952, v50, 286.

4. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*, 1950, v185, 201.

5. Manuscript in preparation.

Received June 23, 1953. P.S.E.B.M., 1953, v83.

Factors Influencing Renin Diuresis and Proteinuria.* (20441)

G. M. C. Masson, Francesco del Greco,† A. C. Corcoran, and Irvine H. Page.

From the Research Division, Cleveland Clinic Foundation, and the Frank E. Bunts Educational Institute, Cleveland, Ohio.

Renin, injected subcutaneously, elicits a variety of abnormal phenomena associated with acute, widespread necrotizing vascular lesions in bilaterally nephrectomized dogs(1) and in uninephrectomized rats pretreated with adrenal cortical steroids(2-5). Administration of excess salt is an essential conditioning factor to the vascular disease elicited by renin. This association and the nature of the lesions suggested that renin might act deleteriously through its effects on electrolyte distribution and on permeability of vessel walls.

In this connection, renin is known to cause increased diuresis and proteinuria in rabbits (6-8) and rats(9-11); the diuresis is associated with changes in electrolyte output(7, 12); the proteinuria is attributed in part to increased permeability of glomerular capillaries(11). The association of renin diuresis with proteinuria was therefore studied as were also the factors which condition these two phenomena since it seemed that such a study might aid in further defining the pathogenetic mechanisms of renin-induced vascular disease.

The particular aspects investigated were: 1) effects of subcutaneous versus intraperitoneal administration; this also involved a comparison of two renin preparations and observation on the effects of subtotal hepatectomy; 2) effects of providing water, sodium chloride and potassium chloride as drinking fluids; 3) effects of adrenalectomy and replacement therapy; 4) effects of hypophysectomy and of ACTH and somatotrophin and 5) effects of large doses of angiotonin.

Procedure. Except as indicated below, the procedure was that male albino rats of uniform strain were placed in metabolism cages 48 hours before injection of renin and kept there for 48 hours after cessation of treatment; urine flow and proteinuria (determined by the Shevky-Stafford method) were measured daily. This general plan, in which each animal is his own control, was required because of the wide variability of urine flow and proteinuria found in these rats as in those studied by Addis *et al.*(9); in some experiments, observations were repeated several times before trends or differences could be established. Since antibodies might interfere with the responses to renin, all animals were discarded from the study after a course of renin treatment.

1. *Route of administration.* The route of administration and the nature of the renin preparation used were of particular interest because of the report(13) that renin given intraperitoneally is much more diuretic than when given subcutaneously. Preliminary experiments had indicated to us that the reverse was the case; the difference in these observations might result from differences in the nonrenin content of the preparations used. Consequently, 2 porcine renin preparations were

---

* This investigation was supported by a grant from the National Heart Institute, National Institutes of Health, Bethesda, Md.

† Post-doctorate Fellow, National Institutes of Health.

TABLE I. Influence of Route of Administration, Nature of Renin Preparation and Salt on Renin Diuresis and Proteinuria.

Group	No. of animals	Avg body wt	Route of administration	Drinking fluid	Avg urine flow in cc/24 hr (a)	(b)	(c)	Avg proteinuria in mg/24 hr (b)
I G	12	203	s.c.	NaCl	20 (18.8–21)	74 (28–158)	27 (20–57)	71.3 (19.6–210)
II G	14	186	i.p.	"	14 (8–25)	47 (3–80)	21 (15–35)	25.5 (2.2–92)
III A	4	205	s.c.	"	18 (9–29)	75 (27–145)	21 (16–25)	99.6 (35–189)
IV A	6	187	i.p.	"	16 (12–25)	32 (10–100)	26 (18–43)	19.8 (2.2–75.5)
V G	10	185	s.c.	Water	8 (5–18)	17 (5–37)	7 (3–10)	3 (1.4–8.6)
VI G	10	183	i.p.	"	6 (5–8)	15 (10–30)	13 (5–16)	10.7 (1.8–30.2)
VII A	6	187	s.c.	"	6 (2–9)	14 (2–30)	6 (5–10)	3.1 (1.7–4)
VIII A	6	187	i.p.	"	6 (5–8)	12 (2–22)	8.8 (4–12)	11.2 (1.5–25)
IX	8	188		NaCl		16 (6–27)		2 (1.1–3.9)
X	9	200		Water		7 (2–12.5)		1.6 (0.6–3.5)

All treated rats (Group I to VIII) received 2 injections of renin (total daily dose 28 dog units). The source of renin is indicated by A (Armour) and G (Green). Values given are: (a) before, (b) during and (c) after, treatment.

studied. One, "G", similar to the one used in previous studies, was prepared by Dr. A. A. Green by isoelectric fractionation with ammonium sulfate, contained 15 mg of protein per cc and had a pressor activity of 1.9 Goldblatt dog units per milligram protein. The other renin, "A", was furnished by Dr. R. J. Seidel, Armour Laboratories, in the form of a powder which had an activity of 0.6 Goldblatt units per mg; this material was suspended in 1% NaCl prior to injections. The effects of subcutaneous and intraperitoneal injections of equipressor doses of both were compared; the dose used was 28 pressor units daily divided in 2 doses and continued over a period of 3 days. The reported difference between intraperitoneal and subcutaneously injected renin might also have been due to some effect of the liver on renin given intraperitoneally. To test this point, observations were made in subtotally hepatectomized animals. The operation consists in ablation of medial, right and left lateral hepatic lobes which amount to about ⅔ of the total organ. The hepatic insufficiency which results from this loss and from the fatty infiltration of the hepatic residue is maximum at about 24 hours postoperatively(14). In this study the animals were placed in metabolism cages immediately after operation and renin injections of the preparation "G" were made both intraperitoneally and subcutaneously.

2. *Effects of salts.* The effects of 1% NaCl and of 1.5% KCl solutions as drinking fluids were compared with those of water in animals given renin "G" and "A" intraperitoneally and subcutaneously.

3. *Adrenalectomy.* Adrenalectomy has been noted to impair renin proteinuria and inhibit the proteinuria of normal rats; adrenal cortical extract partly and cortisone and desoxycorticosterone wholly restore these functions (15). The present experiments were done in order to determine whether the association between renin diuresis and proteinuria persists after adrenalectomy and the effect on both of replacement therapy with hydrocortisone acetate (Hydrocortone, Merck), desoxycorticosterone acetate (DCA) and Lipoadrenal extract (Upjohn). Adrenalectomy was performed in

TABLE II. Influence of Subtotal Hepatectomy and Route of Administration on Renin Diuresis and Proteinuria.

Group	No. of animals	Avg body wt	Route of administration	Avg urine flow in cc/24 hr (b)	Avg urine flow in cc/24 hr (c)	Avg proteinuria in mg/24 hr (b)	Avg proteinuria in mg/24 hr (c)
I	3	459	i.p.	64 (27–115)	21 (12–33)	30.3 (12.2–48.7)	5 (3–7.1)
II	4	443	i.p.	93.8 (16–154)	22 (14–30)	106 (34.5–213)	8.7 (3.5–21.6)
III	4	405	s.c.	56 (7.5–121)	27 (17–31.5)	16.7 (2.5–45)	5.3 (4.5–6.7)
IV	4	425	s.c.	142.5 (99.6–209)	28.8 (17.5–52)	69.5 (29–120)	4.8 (3–9.4)

Rats in Groups I and III were subtotally hepatectomized. Total daily dose of renin: 28 dog units in 2 injections. Drinking fluid: 1% NaCl. (b) and (c) as in Table I.

TABLE III. Influence of Potassium and Sodium Chlorides on Renin Diuresis and Proteinuria.

Group	No. of animals	Avg body wt	Drinking fluid	Avg urine flow in cc/24 hr (a)	Avg urine flow in cc/24 hr (b)	Avg urine flow in cc/24 hr (c)	Avg proteinuria in mg/24 hr (b)
I	6	134	1% NaCl		11 (4–22)		4.5 (1.5–7.9)
II	6	134	1.5% KCl		11 (4–20)		3.1 (1.7–5.8)
III R	6	134	1% NaCl	11 (8–20)	59 (20–100)	18 (17–20)	40 (10.1–86.4)
IV R	6	141	1.5% KCl	11 (8–15)	16 (8–23)	12 (10–15)	4.5 (1.8–9.7)

Renin treated rats (R) received 2 daily subcut. injections totalling 28 dog units. (a), (b) and (c) as in Table I.

two stages, and the animals were maintained on 1% NaCl as drinking fluid during the experimental period. The steroids were given subcutaneously in daily doses of 0.5 or 1.0 mg; hydrocortisone was in saline suspension and DCA in oil. The Lipoadrenal extract was administered subcutaneously in a daily dose of 0.5 cc; this amount had the glycogenic activity of 0.5 mg of cortisone.

4. *Hypophysectomy.* Hypophysectomized rats were obtained from Hormone Assay Laboratories. These were maintained on a high-carbohydrate diet. The hormones tested were ACTH (Armour) and somatotrophin (Antuitrin Growth Hormone, Parke Davis), both in a powder form. ACTH had an activity of 1 LA-1-A unit per mg and Somatotrophin, an activity of 2.5 units per mg. Both were dissolved in 1% NaCl in a concentration of 10 mg per cc and injected intraperitoneally in 5 daily doses of 0.2 cc each.

5. *Angiotonin.* The angiotonin used was prepared by Dr. A. A. Green. Preliminary experiments with a cruder product and with mixtures of incubated renin and renin-substrate indicated that 35 pressor doses did not elicit diuresis or proteinuria.* The preparation used in final experiments was injected intraperitoneally every 30 minutes for 12 hours in a total dose of 247 units. Control animals were given similarly spaced equal volume of fluid (1% NaCl).

Results. 1. *Route of administration.* The data in general indicate a close association between the diuretic and proteinuretic effects of renin (Table I). In confirmation of orienting studies, both responses were greater when renin was given subcutaneously than when it was given intraperitoneally; this was true of both preparations used, in spite of the fact that the greater inactive protein content of renin "A" might have altered its absorption and activity by one route or the other.

---

* Since there is no generally accepted unit of angiotonin, a pressor dose is the amount which causes a rise of 15 mm Hg in blood pressure in an anesthetized dog.

Subtotal hepatectomy impairs the diuretic and proteinuretic responses to renin administered either subcutaneously or intraperitoneally (Table II). The degree to which the operation impairs the response is greater than it would seem to be from the tabulated data; this is because the operation as such (unlisted observations) increases urine flow to a mean of 33 cc and proteinuria to a mean of 9.5 mg per 24 hours during the first postoperative day; these abnormalities receded over the next 48-72 hours.

2. *Water, NaCl and KCl.* The effects of water, 1% NaCl and equimolar KCl on basal diuresis and proteinuria are listed in Table I and III. NaCl alone as compared with water or KCl causes a slight increase in urine flow but no proteinuria. Both renin preparations, whether given intraperitoneally or subcutaneously, elicit greatly enhanced diuresis and proteinuria in rats given NaCl as compared with rats given water or KCl; provision of NaCl causes a greater proportionate increase in renin proteinuria than it does in urine flow; responses to renin in rats given KCl are roughly equal to those observed in animals maintained on water.

3. *Adrenalectomy.* The observations (Table IV) confirm the fact(15) that adrenalectomy inhibits renin proteinuria; supplemental observations are that adrenalectomy similarly inhibits the diuretic response and that these inhibitory effects are not restored by provision of 1% NaCl. Lipoadrenal extract alone, in the dose used, did not affect urine flow or proteinuria; however, replacement therapy with this extract, or with DCA or hydrocortisone all restored diuretic and proteinuretic responses to renin. The spread of the data is such that no decision can be reached as to the relative effects of the 2 steroids or of the 2 dose levels used.

4. *Hypophysectomy.* This operation, like adrenalectomy impairs diuretic and proteinuretic responses to renin, (Table V). Because somatotrophin has been observed to restore renal function and water diuresis in hypophysectomized dogs(16,17), it seemed likely that

TABLE IV. Influence of Adrenalectomy and Replacement Therapy on Renin Diuresis and Proteinuria.

Group and renin treatment		No. of animals	Avg body wt	Adrenal steroid and dose/day	Avg urine flow in cc/24 hr (a)	(b)	(c)	Avg proteinuria in mg/24 hr (a)	(b)	(c)
I	0	6	156	0		15			3.6	
II	0	5	159	L.A.E.	10.1 (5.5-14.5)	14 (8–29)	17 (11–21)	2.4 (0.9–4)	3.1 (0.6–9.1)	3 (2.1–4.5)
III	R	9	160	0	11.6 (7.5–22)	26 (9–44)	22 (9–42)	3.5 (1.4–6.3)	7 (1.3–15)	4.1 (1.2–14.4)
IV	R	6	162	L.A.E., .5 cc	12.9 (8–25)	62 (27–106)	20 (12–30)	2.3 (0.6–5.4)	34.2 (8.4–93.6)	6.1 (1.2–8.6)
V	R	6	157	Hydrocortisone, 1 mg	16.5 (9–21)	39.1 (13–81)	22 (17–45)	3 (.14–6)	102 (20.6–233)	3.6 (1.7–4.5)
VI	R	6	154	Hydrocortisone, .5 mg	8.1 (6–11)	31 (14–82)	32.7 (2.8–43)	2.7 (1.8–4.4)	18 (2.4–92)	5.1 (2.3–10.8)
VII	R	5	157	DCA, 1 mg	23 (18.5–26)	31.4 (19–55)	23 (9–49)	4 (1.5–5.8)	17.6 (15–19.4)	3.8 (1.6–14.7)
VIII	R	2	168	DCA, .5 mg	8.5 (5–12)	64 (25–103)	40 (39–40)	2.4 (1.5–3.5)	14.2 (2.6–48.2)	7.1 (2.8–11.5)

Dose of renin (R) was 28 dog units in 2 subcut. injections. Drinking fluid: 1% NaCl. (a), (b) and (c) as in Table I.

TABLE V. Influence of Hypophysectomy and Pituitary Hormones on Renin Diuresis and Proteinuria.

Group and renin treatment	No. of animals	Avg body wt	Pituitary hormone and daily dose	Avg urine flow in cc/24 hr	Avg proteinuria in mg/24 hr.
I 0	6	145	0	13.3 (1–37.5)	1.3 (.1–2.7)
II R	6	147	0	17 (5.2–47.5)	2.1 (.4–30.3)
III R	4	138	ACTH 5 × 2 mg	38.2 (5–83.6)	13.9 (3.7–33)
IV R	4	145	STH 5 × 2 mg	12 (1–39.5)	2.7 (.4–6.9)
V 0	5	137	STH 5 × 2 mg	7 (1–12)	1.3 (.2–2.6)
VI 0	10	138	ACTH 5 × 2 mg	32 (11–72)	28.7 (4–67)

Daily dose of renin (R) was 28 dog units in 2 subcut. injections. Drinking fluid: 1% NaCl.

TABLE VI. Effects of Angiotonin on Diuresis and Proteinuria.

Group and treatment	No. of animals	Avg body wt	Avg urine flow in cc/24 hr (a)	(b)	Avg proteinuria in mg/24 hr (a)	(b)
I Angiotonin	3	203	18.3 (11.6–27)	45 (40–49)	2.3 (1–3.5)	49.6 (14.4–102)
II 0	3	233	22.9 (19–30)	24 (15.2–32)	5.5 (2.2–8.1)	8 (3.3–17.3)

Total dose of angiotonin: 247 pressor doses in 24 intraperitoneal injections. Drinking fluid: 1% NaCl. (a) and (b) as in Table I.

it might restore responses to renin; this, under our experimental conditions, was not the case. The observations in adrenalectomized rats given replacement therapy suggested that ACTH would restore the renin responses of hypophysectomized animals; however, in the dose used, ACTH was diuretic and proteinuretic of itself, and additive responses to renin were negligible.

5. *Angiotonin.* The observations are summarized in Table VI; these demonstrate that, in the dose used, intraperitoneally injected angiotonin increases both urine flow and proteinuria.

Discussion. The observations demonstrate the close association of diuresis and proteinuria as responses to injection of renin and, since these responses also follow on injection of angiotonin, reaffirm Addis' conclusion(9) that these urinary changes are integral responses to the activity of the renal pressor system. Both responses to renin are impaired by adrenalectomy and restored by replacement therapy; both are similarly impaired by hypophysectomy, and somatotrophin does not restore them, while, in the doses used, ACTH caused diuresis and proteinuria which obscured any additive effect of renin.

In contrast to the observations of Croxatto (13) the diuretic and also the proteinuretic effect of renin was found to be greater when the renin was injected subcutaneously than when it was given intraperitoneally. This was true of two quite different renin preparations tested in equipressor doses, so that the difference between our results and theirs remains unexplained.

Subtotal hepatectomy was found to impair diuretic and proteinuretic responses to renin; this may reflect defective formation of renin-substrate or, as has been suggested, liberation of endogenous renin in response to operative trauma(18). The diuresis and proteinuria observed during the first 24 hours after operation are consistent with the latter view.

Potentiation of renin diuresis by administration of NaCl solution as drinking fluid had been previously observed(13). This potentiation is shown to apply also to postrenin proteinuria; KCl is shown to be ineffective. Thus, the sensitization to the diuretic and proteinuretic effects of renin is dependent on provision of sodium as such; renin-induced acute vascular diseases are similarly dependent on administration of sodium and of steroids which affect sodium distribution; thus, it may be that renin diuresis and proteinuria are in normal animals, aspects of general phenomena which under specific conditions precipitate acute vascular lesions.

Summary. 1. Administration of renin elicits increases in urine flow and urine protein in rats; these phenomena are closely associated; both are impaired by adrenalectomy or hypophysectomy and restored, after adrenalectomy, by replacement therapy; under the experimental conditions neither somatotrophin nor ACTH could be shown to restore the impaired renin responses of hypophysectomized rats. 2. The two different renin preparations used were more effective in stimulating urine flow and proteinuria when given subcutaneously than when given intraperitoneally. 3. Subtotal hepatectomy impaired these responses to injected renin. 4. The administration of sodium potentiates the diuretic and proteinuretic effects of injected renin. 5. These responses can be paralleled by appropriate intraperitoneal administration of angiotonin.

The authors are indebted to Dr. E. Alpert, Merck and Co., for supplies of cortisone and hydrocortisone; Dr. H. Hailman, Upjohn Co., for Lipoadrenal extracts; Dr. K. Thompson, Organon Inc. for desoxycorticosterone; Dr. R. J. Seider, Armour Laboratories, for a renin preparation and Dr. E. C. Vonder Heide, Parke, Davis and Co., for antuitrin growth hormone.

1. Masson, G. M. C., Plahl, G., Corcoran, A. C., and Page, I. H., *A. M. A. Arch. Path.*, 1953, v55, 85.

2. Masson, G. M. C., Corcoran, A. C., and Page, I. H., *J. Lab. Clin. Med.*, 1951, v38, 213.

3. ———, *A. M. A. Arch. Path.*, 1952, v53, 217.

4. Masson, G. M. C., del Greco, F., Corcoran, A. C., and Page, I. H., *A. M. A. Arch. Path.*, 1953, v53, 23.

5. ———, *Am. J. Med. Sc.*, 1953, in press.

6. Pickering, G. W., and Prinzmetal, M., *J. Physiol.*, 1940, v98, 314.

7. Hughes-Jones, N. C., Pickering, G. W., Sanderson, P. H., Scarborough, H., and Vandenbroucke, J., *J. Physiol.*, 1949, v109, 288.

8. Brandt, J. L., and Gruhn, J. G., *Am. J. Physiol.*, 1948, v153, 458.

9. Addis, T., Barrett, E., Boyd, R. I., and Ureen, H. J., *J. Exp. Med.*, 1949, v89, 131.

10. Masson, G. M. C., Corcoran, A. C., and Page, I. H., *Am. J. Physiol.*, 1950, v162, 379.

11. Sellers, A. L., Smith, S., III, Marmorston, J., and Goodman, H. C., *J. Exp. Med.*, 1952, v96, 643.

12. Sellers, A. L., Smith, S., III, Goodman, H. C., and Marmorston, J., *Am. J. Physiol.*, 1951, v166, 619.

13. Croxatto, H., Barnafi, L., and Passi, J., *Science*, 1952, v116, 507.

14. Masson, G. M. C., and Beland, E., *Anesthesiology*, 1945, v6, 483.

15. Addis, T., Marmorston, J., Goodman, H. C., Sellers, A. L., and Smith, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 43.

16. White, H. L., Heinbecker, P., and Rolf, D., *Am. J. Physiol.*, 1949, v157, 47.

17. de Bodo, R. C., Schwartz, I. L., Greenberg, J., Kurtz, M., Earle, D. P., Jr., and Farber, S. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 612.

18. Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., Munoz, J. M., and Taquini, A. C., *Renal Hypertension*, Springfield, Ch. C. Thomas, 1946.

Received June 24, 1953. P.S.E.B.M., 1953, v83.

Nitrogen Metabolism of Isolated Muscle of Normal and Vitamin E-Deficient Hamsters.* (20442)

ANNE E. MILMAN, DEMETRIA N. SILIDES AND A. T. MILHORAT.

From the Departments of Psychiatry and Medicine, Cornell University Medical College, the Russell Sage Institute of Pathology, and The New York Hospital, New York.

In muscular dystrophy resulting from Vit. E-deprivation, there is a loss of muscle substance from the animal body suggesting that the rate of protein breakdown is considerably accelerated. The purpose of this paper is to report the results of experiments with isolated tissues in which the rate of protein catabolism, as judged by the rate of accumulation of amino nitrogen, was compared in normal and in dystrophic tissues. The present findings suggesting actual diminution in protein breakdown are of interest.

Materials and methods. For this purpose, the method devised by Kline(1) for studying nitrogen metabolism in isolated tissue slices incubated in an artificial medium was used. This method is based on the estimation of the loss of nitrogen from tissue slices during incubation by determination of the increase of nitrogen in the bicarbonate buffer medium. During incubation there is always a loss of nitrogen from the tissues, and Frantz, Loftfield and Miller(2) using labelled nitrogen found that under *in vitro* conditions the breakdown of protein exceeds protein synthesis. However, these authors concluded, as had Bernheim and Bernheim(3) previously, that the rate of loss of nitrogen gives important information regarding the nitrogen metabolism of the tissues. Kline studied slices of liver and diaphragm from normal, hypophysectomized, thyroidectomized and adrenalectomized rats, and found alterations in the rates of nitrogen metabolism that were reproducible and compatible with the changes which the removal of these glands is known to induce in the intact animal. Moreover, the validity of the method as an index of active metabolism rather than merely a measure of diffusion or other purely physical phenomena, was demonstrated further by the study of the restorative effects of administration of the hormones of the thyroid and adrenals on the metabolism of tissues of animals deprived of these glands.

Young hamsters weighing between 90 and 125 g were used. One group of animals (normal group) were fed the deficient diet[†] plus an adequate supplement of Vit. E. A second group were maintained on the Vit. E-deficient diet for periods of from 4 to 6 weeks, after which time, microscopic studies of the skeletal muscles from a representative number of animals showed structural changes typical of muscular dystrophy. After the animals had been anesthetized with nembutal, the gastrocnemii and diaphragm were removed and chilled immediately. Slices weighing from 30 to 100 mg were placed in test tubes containing 4 ml of Krebs-Ringer bicarbonate buffer and 0.2% glucose. The solution was saturated with O_2 just before and after the addition of the tissue. The tubes were placed in a mechanical shaker immersed in a water bath at 37°C and shaken at a rate of about 120 times per minute.

At the end of a 15 minute equilibration period, some of the tubes were removed to serve for zero value determinations as described by Kline. Triplicates of diaphragm and of skeletal muscle were used for establishing this base line and another 3 samples of each were used for comparison at the end of one hour of incubation. Proteins were precipitated from the medium with tungstic acid. Free amino nitrogen was then determined colorimetrically(4). The slices varied considerably in weight because they were prepared and weighed rapidly. However, differences within the range used in these experiments did not influence the results.

Results and discussion. The results of these experiments are summarized in the table, and

---

* Aided by grants from the Muscular Dystrophy Associations of America, Inc. and the Association for the Aid of Crippled Children.

[†] % content of diet: sucrose 20; casein 7; rolled oats 40; skimmed milk powder 29; sodium chloride 1; lard 1; cod liver oil 2.

TABLE I. Release of Amino Nitrogen from Isolated Skeletal Muscle and Diaphragm of Normal and Dystrophic Hamsters.

Tissue	No.	Amino-N in medium (μg/100 mg wet wt) 15 min.	60 min.	Difference
Skeletal muscle, normal	15	17.8	50.7	33.0 ± 1.7
" " vit. E-def.	12	13.3	41.1	27.9 ± 2.8
Diaphragm, normal	15	20.2	52.2	32.0 ± 2.2
" vit. E-def.	12	17.3	42.9	25.8 ± 3.1

are expressed as μg of amino nitrogen per 100 mg of tissue (wet weight). Observations on a representative number of samples, showed no significant difference in the water content of the tissues of normal and Vit. E-deficient animals. It was found that both the "initial" and final amounts of amino nitrogen in the medium were greater in the experiments with tissues of normal animals than with tissues of Vit. E-deficient animals. The differences are statistically significant although not numerically large. It should be pointed out, however, that they have been calculated on Kline's assumption that the differences between the 15 min. values and those obtained later (*i.e.* 60 min.) are more significant than are the absolute values at the end of the incubation. If these final values were equally significant, it can be seen that the differences would be greater. In the opinion of Kline, the amount of nitrogen which enters the medium during the first 15 minutes is determined largely by the effects of slicing, tissue damage and the surface area of the slice, and may not be a good indication of the metabolic processes of the tissue. For this reason, the end of this 15-minute incubation period is taken as "zero time." However, this does not explain our observation that dystrophic tissues invariably yielded lower 15 min. values than did the normal tissues.

In 2 experiments in which skeletal muscle of normal and of Vit. E-deficient rabbits were compared, essentially similar results were obtained.

If the metabolic activity of these surviving slices actually is an indication of the relative metabolic rates of the tissues of these animals *in vivo*, these results would suggest that the rate of protein breakdown in the dystrophic animal may not be enhanced. On the contrary, it is possible that the rate of protein catabolism may be reduced, and that this reduction is secondary to a decreased rate of protein synthesis. Obviously, this possibility should be investigated further by other methods, since it offers an entirely new concept in our consideration of the mechanism of the loss of muscle that occurs in muscular dystrophy.

Summary. A significant decrease was observed in the rate of release of amino nitrogen by the slices of surviving diaphragm and skeletal muscle from Vit. E-deficient hamsters when compared with tissues of normal controls.

---

1. Kline, D. L., *Endocrinology*, 1949, v45, 596.
2. Frantz, I. D., Loftfield, R. B., and Miller, W. W., *Science*, 1947, v106, 544.
3. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, 1946, v163, 203.
4. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, 1943, v149, 255.

Received June 26, 1953. P.S.E.B.M., 1953, v83.

Hypothermia in Experimental Hepatic Surgery.* (20443)

FRANCISCO L. RAFFUCCI,† F. JOHN LEWIS, AND OWEN H. WANGENSTEEN.
From the Department of Surgery, University of Minnesota Medical School, Minneapolis.

Hypothermia has been used recently(1-5) to perform intracardiac operations under direct vision without recourse to the use of extracorporeal blood pumps. The success of this technic is due to the greatly lowered oxygen requirements at reduced temperatures which allow a relatively long period of total inflow occlusion. The brain, which is the crucial organ during the cardiac operations, can apparently withstand anoxia about twice as long at a temperature of 26°C as it can at the normal body temperature. Presumably other organs as well could tolerate longer periods of anoxia during hypothermia, and may in this state, after temporary local vascular occlusion, be subjected to longer bloodless operations than could be attempted with safety at normal temperatures. If successful, this use of hypothermia might have practical value as an aid in performing extensive liver resections.

For surgery of the liver, the advantage of a bloodless field provided by prolonged complete vascular occlusion is obvious. It has been our experience, however, to find that complete hepatic vascular occlusion is not tolerated long at normal body temperatures (6,7). Hence, we have investigated the efficacy of hypothermia in allowing vascular occlusion of a satisfactory duration. At normal body temperatures the maximum period of continuous hepatic artery and portal vein occlusion tolerated by dogs without resulting in some deaths was found to be 20 minutes. After this, as the period of anoxia was lengthened, the fatality rate increased so that when the period of occlusion was extended to one hour there was only one survivor in 49 experiments. This high fatality rate occurred in spite of the fact that 46 of these dogs received antibiotics to prevent growth of the anaerobic bacteria which normally inhabit the canine liver(8-11).

Methods and results. The small, mongrel, adult dogs used in these experiments were given a combination of antibiotics for 48 hours prior to surgery. Aureomycin was given orally twice a day in 500 mg doses while penicillin was given intramuscularly twice daily in doses of 150,000 units. After fasting for 12 hours, the dogs were anesthetized with a 2.5% solution of sodium pentothal intravenously and cooling was started. Anesthesia was maintained to prevent shivering but when the temperature had fallen to 28°C no further anesthesia was needed. After the body temperature had reached 30°C the dogs were given artificial respiration by means of an electronic respirator. Body temperature was measured by means of a rectal thermometer inserted to a distance of 3 inches.

Each dog was cooled by wrapping the entire animal in rubberized blankets through which flowed a cold alcohol solution at a temperature slightly above the freezing point of water. Postoperatively, warming was accomplished with the same blankets, the circulating solution having been heated.‡

The operation was begun when the animal's temperature had dropped to 28°C or lower. Using aseptic technic, the first step was to clamp the celiac axis and the superior mesenteric artery to prevent the intestinal engorgement which would otherwise follow the subsequent venous occlusion. Then, within one minute, the hepatic artery was occluded distal to the point of origin of the gastroduodenal artery and the portal vein was occluded just before it entered the liver. The period of vascular interruption was one hour, after which the clamps were removed, the abdomen was closed, and the animal warmed as described.

Eleven dogs were submitted to the above

---

* Supported in part by a Grant-in-Aid from the Graduate School of the University of Minnesota and from the following additional sources: The Augustus L. Searle Fund, The Austen S. Cargill Fund, and The Malignant Disease Research Fund.

† Now a member of the Department of Surgery, Medical School, San Juan, P. R.

‡ The cooling apparatus was manufactured by the Therm-O-Rite Products Corporation, Buffalo, N. Y.,

TABLE I. Effect of Hypothermia on Liver Anoxia.

Dog No.	Lowest body temp.	Results		Autopsy findings
		Died	Survived	
1	22°C	×		Liver necrosis
2	25°C	×		Hemorrhage in liver cords
3	28°C		×	Normal liver when sacrificed at 21st postoperative day
4	28°C	×		” ” microscopically
5	26°C		×	” ” when sacrificed at 20th postop. day
6	26°C		×	” ” ” ” ” 20th ” ”
7	26°C		×	” ” ” ” ” 14th ” ”
8	25°C		×	Died at 21st postoperative day with unexplained abdominal abscess. Liver was normal
9	26°C		×	Died at 21st postoperative day of pneumonia. Liver was normal
10	26°C		×	Normal liver when sacrificed at 21st postoperative day
11	26°C		×	” ” ” ” ” 56th ” ”

procedure and the results are tabulated in Table I. Animals surviving the operation for a period of 2 weeks are listed as survivors since our previous experience with the control animals(7) indicated that dogs dying of liver necrosis following complete vascular occlusion usually died within the first 2 postoperative days.

Discussion. Previously we reported a fatality rate of 100% among 46 dogs submitted to occlusion of the total afferent hepatic circulation when antibiotics were administered (7). This fatality rate was reduced by the preoperative administration of the adrenocorticotropic hormone obtained from hog pituitaries so that, among 36 animals so treated, 17 survived. The factors that might have led to this increased survival rate in the treated animals have been discussed in the earlier paper.

The present experiments indicate that hypothermia, too, was effective in reducing the fatality rate which followed afferent hepatic vascular occlusion maintained for one hour. The influence of hypothermia is, as was mentioned earlier, doubtless due to its effect in reducing the metabolic activity of the liver. Our experiments do not indicate whether corticotrophin or hypothermia is more competent in reducing the toxic effects of hepatic vascular occlusion and we have not yet tested the efficacy of using them simultaneously. The experimental evidence presented suggests, however, that one, or the other, or a combination may prove to have a practical clinical value in facilitating massive resections of the liver.

Summary. When hypothermia is induced in dogs prior to the production of liver anoxia by completely occluding the afferent hepatic circulation, the mortality rate of the procedure is greatly reduced (27.2%) as compared to that previously reported in control animals (100%).

---

1. Bigelow, W. G., Lindsay, W. K., Harrison, R. C., Gordon, R. A., and Greenwood, W. F., *Am. J. Physiol.*, 1950, v160, 125.

2. Bigelow, W. G., Lindsay, W. K., and Greenwood, W. F., *Ann. Surg.*, 1950, v132, 849.

3. Boerema, I., Wildschut, A., Schmidt, W. J. H., and Broekhuysen, L., *Arch. chir. Neerl.*, 1951, v3, 24.

4. Cookson, B. A., Neptune, W. B., and Bailey, C. P., *Diseases of the Chest*, 1952, v22, 245.

5. Lewis, F. J., and Taufic, M., *Surgery*, 1953, v33, 52.

6. Raffucci, F. L., and Wangensteen, O. H., *Surgical Forum*, W. B. Saunders Co., Philadelphia, 1950, 191-195.

7. Raffucci, F. L., *Surgery*, 1953, v33, 342.

8. Markowitz, J., Rappaport, H., and Scott, A. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 305.

9. Markowitz, J., and Rappaport, A. M., *Physiol. Rev.*, 1951, v31, 188.

10. Fitts, W. T., Scott, R., and Mackie, J. A., *Surgery*, 1950, v28, 458.

11. Lewis, F. J., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 533.

Received October 30, 1952. P.S.E.B.M., 1953, v83.

Protection of Embryonated Eggs Infected with Infectious Bronchitis or Newcastle Disease Virus by Polypeptides.[*] (20444)

MAURICE GREEN, M. A. STAHMANN, AND A. F. RASMUSSEN, JR.[†]

From the Departments of Biochemistry and Medical Microbiology, University of Wisconsin, Madison.

Studies on the antiviral properties of synthetic lysine polypeptides have revealed them to inhibit tobacco mosaic virus infectivity(1), to inhibit the growth of influenza virus in the chick embryo(2), and to possess antiviral activity against a bacteriophage(3). A report on the protective effect of lysine polypeptides on embryonated eggs infected with several strains of infectious bronchitis virus and also Newcastle disease virus is presented here.

Materials and methods. The L-lysine polypeptide hydrochloride of molecular weight 4900 was supplied by Dr. R. R. Becker(4). This was dissolved in physiological saline and adjusted to pH 6.3. Solutions of protamine sulfate, lysozyme and L-lysine were prepared in a similar manner. The glutamic acid polypeptides(5) were dissolved in an equivalent amount of alkali and adjusted to pH 7.3. All solutions were passed through a Seitz filter and tested for bacterial sterility. The viruses[‡] employed in this study included 4 strains of infectious bronchitis virus of chickens (IBV) as follows: (a) a recently isolated Wisconsin strain, (b) the Jungherr strain, (c) the egg adapted strain of Beaudette and Hudson, and (d) the egg adapted Van Roekel strain; as well as the Newcastle disease virus, New Jersey K. D. strain. Inoculum pools of the various viruses were prepared by allantoic sac passage. Infectious bronchitis virus was stored in inactivated normal horse serum at –70°C to maintain its titer. The infectivity titers of the viral inocula were determined by inoculation of serial decimal dilutions of the virus pools into groups of 6 eggs each. One-tenth or 0.2 ml amounts of the materials to be tested for their protective effect were injected into the allantoic sac of eggs 1 hour before or after challenge with virus by the same route. Infectious bronchitis virus and Newcastle disease virus were inoculated into the allantoic sac of eggs in the 9th and 11th day of embryonic development respectively. All eggs were incubated at 37°C and candled

TABLE I. Effect of Polypeptides in Allantoic Sac of Embryonated Eggs Infected with Infectious Bronchitis Virus (IBV).

1st inj.	2nd inj. (1 hr later)	Dead/ total[‡]	Avg day of death
A. Wisconsin Strain—early egg passage[§]			
mg			
10 PGA[*]	10 LD_{50} IBV	9/9	2.7
0.2 PL[†]	” ” ”	4/9	5.3
Saline	” ” ”	8/8	2.7
10 PGA	Saline	0/5	—
0.2 PL	”	0/8	—
0.04 PL	10 LD_{50} IBV	4/10	3.5
10 LD_{50} IBV	0.2 mg PL	2/9	—
Saline	10 LD_{50} IBV	7/9	2.4
0.2 PL	100 LD_{50} IBV	2/10	—
Saline	” ” ”	9/9	2.4
0.2 PL	1000 LD_{50} IBV	2/9	—
Saline	” ” ”	10/10	2.4
B. Wisconsin Strain—15-17th egg passage			
0.2 PL	100 LD_{50} IBV	11/20	3.1
0.2 Protamine	” ” ”	16/19	4.6
Saline	” ” ”	16/18	2.8
0.2 L-Lysine	100 LD_{50} IBV	9/10	2.3
0.5 Lysozyme	” ” ”	6/8	2.7
Saline	” ” ”	8/8	2.6
C. Jungherr Strain—7th egg passage			
0.2 PL	100 LD_{50} IBV	7/11	4.1
Saline	” ” ”	11/11	3.4
0.2 PL	10 LD_{50} IBV	2/11	—
0.2 Protamine	” ” ”	6/11	4.1
Saline	” ” ”	9/12	3.6

[*] PGA—Polyglutamic acid of avg molecular wt 4800.

[†] PL—Polylysine of avg molecular wt 4900.

[‡] Computed for deaths 6 days after inoculation.

[§] The exact passage not known.

---

[*] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a research grant from the National Institutes of Health, U. S. Public Health Service.

[†] Present address: Department of Infectious Diseases, School of Medicine, University of California, Los Angeles.

[‡] Our thanks are due to Dr. R. P. Hanson for generous advice and for cultures of the Van Roekel and Jungherr strains of infectious bronchitis virus, Miss E. Upton for a culture of the Wisconsin strain, and Dr. V. Groupe for the Beaudette and Hudson strain.

TABLE II. Effect of Polypeptides in Allantoic Sac of Embryonated Eggs Infected with Egg-Adapted Infectious Bronchitis Virus (IBV).

1st inj.	2nd inj. (1 hr later)	Dead/ total	Avg day of death
	A. Beaudette and Hudson Strain		
mg			
0.4 PL*	10 LD_{50}	3/8	—
1.8 PGA†	” ”	5/5	2.3
Saline	” ”	7/9	1.5
0.4 PL	100 LD_{50}	9/9	1.5
5 Lysozyme	” ”	8/8	1.5
Saline	” ”	8/8	1.7
	B. Van Roekel Strain		
0.4 PL	10 LD_{50}	13/13	1.6
Saline	” ”	13/14	1.5

* PL—Polylysine of avg molecular wt 4900.

† PGA—Polyglutamic acid of avg molecular wt 4800.

TABLE III. Activity of Polypeptides in Allantoic Sac of Embryonated Eggs Infected with Newcastle Disease Virus (NDV).

1st inj.	2nd inj. (1 hr later)	Dead/ total	Avg day of death
mg			
2 PGA*	100 LD_{50} NDV	8/8	2.3
0.2 PL†	” ” ”	7/7	2.4
0.2 Protamine	” ” ”	7/7	2.4
Saline	” ” ”	8/8	2.3
0.4 PL	6 LD_{50}	8/15	3.1
5 Lysozyme	” ”	10/10	2.5
Saline	” ”	13/14	2.7

* PGA—Polyglutamic acid of avg molecular wt 15600.

† PL—Polylysine of avg molecular wt 4900.

daily or twice daily for 6 days after infection (6). The criterion of antiviral activity was the survival of the embryo.

Experimental and results. The maximum non-lethal dose of the lysine polypeptide and protamine sulfate per embryo was 1 and 0.5 mg respectively. Lysozyme and glutamic acid polypeptides were not toxic at 5 mg and 15 mg per embryo respectively.

Some typical results of screening experiments carried out with several strains of infectious bronchitis virus are presented in Tables I and II. Table I-A summarizes the results obtained with an early egg passage of a strain of infectious bronchitis virus isolated in Wisconsin which was still pathogenic to chickens. As little as 0.2 mg of polylysine when injected 1 hour prior to inoculation with from 100 to 1000 LD_{50} of virus allowed 80% of the embryos to survive whereas all the control embryos were dead within 6 days. Injection of polylysine 1 hour after inoculation with 10 LD_{50} of virus also showed a marked protective effect. Four hundredths mg of polylysine was sufficient to protect against 10 LD_{50} of virus. Polyglutamic acid of the same molecular weight showed no protection. Table I-B reveals that inhibition by polylysine of infectious bronchitis virus is diminished with increased egg passage. Thus in several experiments only 30-50% of the embryos survived 100 LD_{50} of virus passed 15 to 17 times when treated with 0.2 mg of polylysine as compared with 80% survival with the earlier passage of the virus.

Three other strains of infectious bronchitis virus were screened for inhibition by the polypeptides to further test the suggestion that with increased egg passage the virus becomes more resistant to inhibition by polylysine. They were the Jungherr strain which had been through 7 egg passages, the Beaudette and Hudson strain which had been through several hundred egg passages and was non-pathogenic to birds, and the Van Roekel strain which had been through at least a hundred egg passages. Table I-C reveals that significant protection of embryos infected with 10 and 100 LD_{50} of the Jungherr strain was obtained. Table II-A shows that the Beaudette and Hudson strain was inhibited at the 10 LD_{50} level but not at 100 LD_{50}. No protection by polylysine was observed in eggs infected with 10 LD_{50} of the Van Roekel strain as shown in Table II-B.

Polylysine protected eggs against 6 LD_{50} of Newcastle disease virus as shown in Table III. No protection was observed against 100 LD_{50} of the virus. As much as 5 mg of lysozyme had no protective action.

Discussion. Polylysine was found to protect embryonated eggs infected with infectious bronchitis virus. Protamine also afforded a small amount of protection while lysozyme had little or no effect. The data on the 4 strains of infectious bronchitis investigated suggest that with increased egg passage the virus becomes more resistant to polylysine ininhibition. The virus also loses its antigenicity and becomes less pathogenic to birds with increased egg passage(7). Polylysine has been shown to inhibit tobacco mosaic virus infectivity by direct combination with the virus(8). It is therefore suggested that polylysine inhibits infectious bronchitis virus by a similar combination of the basic polypeptide with acidic groups on the surface of the virus protein. With egg passage, the virus becomes less antigenic, the surface pattern of the virus protein is modified perhaps in such a manner as to render it less available for combination with polylysine. It is also possible that the resistance to polylysine inhibition with increased egg passage reflects only an increase in the pathogenicity of the virus.

Conclusion. Polylysine was found to exert a marked protective effect in embryonated eggs subsequently infected with recently isolated infectious bronchitis virus. This protective effect was significantly less after several egg passages and with strains of infectious bronchitis which were completely egg adapted. Embryonated eggs infected with Newcastle disease virus were also protected by polylysine.

---

1. Stahmann, M. A., Graf, L. H., Patterson, E. L., Walker, J. C., and Watson, D. W., *J. Biol. Chem.*, 1951, v189, 45.
2. Rubini, J. R., Rasmussen, A. F., and Stahmann, M. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 662.
3. Watson, D. W., and Bloom, W. L., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 29.
4. Becker, R. R., and Stahmann, M. A., *J. Am. Chem. Soc.*, 1952, v74, 38.
5. Green, M., and Stahmann, M. A., *J. Biol. Chem.*, 1952, v197, 771.
6. Groupe, V., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 354.
7. Loomis, L. N., Cunningham, C. H., Gray, M. L., and Thorp, F., Jr., *Am. J. Vet. Res.*, 1950, v11, 245.
8. Burger, W. C., and Stahmann, M. A., *J. Biol. Chem.*, 1951, v193, 13.

Received May 18, 1953. P.S.E.B.M., 1953, v83.

Application of Tissue Clearance Technics in Small Animals.* (20445)

CHESTER HYMAN, ALFRED STONE, AND MAX SOSNOW.

From the Department of Physiology, School of Medicine, University of Southern California. Los Angeles.

Careful work in many laboratories has established the fact that the disappearance of an isotope from the site of local injection is an exponential function. In his original analysis of the dynamics of tissue clearance, Kety(1) postulated that such an exponential relationship should obtain, if one could assume that the arterial concentration of the tracer were small enough to be neglected. That this assumption is substantially true in the case of clearance from the tissue of man has been amply confirmed in many laboratories(2-7). A most stringent test of the validity of this assumption is the linearity of the disappearance curve over long periods of time; thus, McGirr(3) and Rapaport *et al.*(4) find no significant deviation from linearity in disappearance curves followed for more than 60 minutes. However, data obtained in similar experiments with small animals frequently yield curves which are not strictly exponential. This is especially evident where the disappearance is followed for periods greater than 20 or 30 minutes. Thus, in short term investigations (8-10), *i.e.*, 5 to 10 minutes, the clearance of radioisotopes from various sites of injection in rats, rabbits and dogs was quite linear. When the period of observation was extended beyond 30 minutes deviations from the simple exponential relationship were always found(9, 10).

---

* This investigation was supported by a research grant from the National Heart Institute of the National Institutes of Health, Public Health Service; and by a grant from the Los Angeles County Heart Association.

The radioisotopes used in this investigation were supplied by Oak Ridge National Laboratories, Oak Ridge, Tenn. and obtained on allocation from the U. S. Atomic Energy Commission.

The possible sources of this non-linearity have been considered by Cooper *et al.*(5), Miller *et al.*(6), and Wisham *et al.*(7). These factors include: a) a continually changing blood flow in the tissue; b) the deposition of significant amounts of the tracer in areas where the rate of removal was slower than from the bulk of the injection; and c) an increase in the concentration of the isotope in the arterial blood returning to the original injection site, *i.e.*, a negation of Kety's assumption. In the present study, we have attempted to determine which of these factors is the most significant and to devise methods to compensate for the deviations observed.

Methods. All clearances were measured on pentobarbital anesthetized rabbits. About 10-15 μC of I^{131} as iodide or Na^{24} as sodium chloride were injected in a total volume of 0.5 ml of isotonic saline. All injections were made into the subcutaneous tissue on the medial aspect of the thigh at least 24 hours after shaving the skin. In all cases small short needles were used. An attempt was made to keep the depth of injection, the length of the needle, the amount of solution and the geometry of the counting tubes constant. In addition, environmental factors were kept as nearly identical as possible for each rabbit: room temperature, duration of the experiment and depth of anesthesia were carefully controlled. All of our experiments were continued for at least 30 minutes. A) If the change in clearance reflects a change in blood flow attendant on anesthesia or on the abnormal position of the animal, we assumed that these factors would ultimately achieve a steady state and become relatively constant, or if variations continued, they should become more random and show increases as well as decreases. Therefore, clearances were determined in some animals for a 30 to 60 minute period immediately after induction of anesthesia; in others, clearances of similar duration were determined beginning about an hour after anesthesia was begun; and in a third group clearances were followed continually for a period of more than 3 hours beginning soon after induction of anesthesia. B) Another possible explanation of the deviation from linearity might be the deposition of some radioisotope along the needle track, involving several layers of tissue having different blood-flow rates. This possibility was explored by making subcutaneous injections with a long needle (3″) introduced through the skin at a point remote from the site of injection at the final position of the tip. As an added precaution, the needle was emptied of all isotope solution by passing a small air bubble through just prior to withdrawal. C) Finally, we investigated the possibility that significant quantities of the isotope were being returned via the arterial blood to the area "seen" by the counter over the injection site. To accomplish this, we placed a second counter over a control area on the contralateral limb in a position corresponding to the counter over the injection site. Lead bricks were arranged in such a way as to minimize cross-counting. The activity recorded by the G. M. Counter over the uninjected limb was taken to represent the contribution of all the counts brought by the arterial blood into this area and hence into the corresponding area on the injected leg. The difference between the two counters then should represent

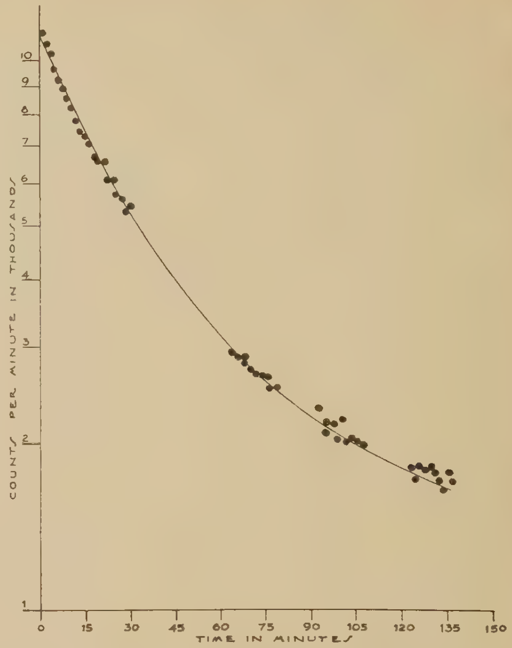


FIG. 1. Clearance of I^{131} from subcut. tissue of rabbit. The animal was anesthetized just prior to injection at zero time and the counting rate over injection site was determined for the following 2½ hr.

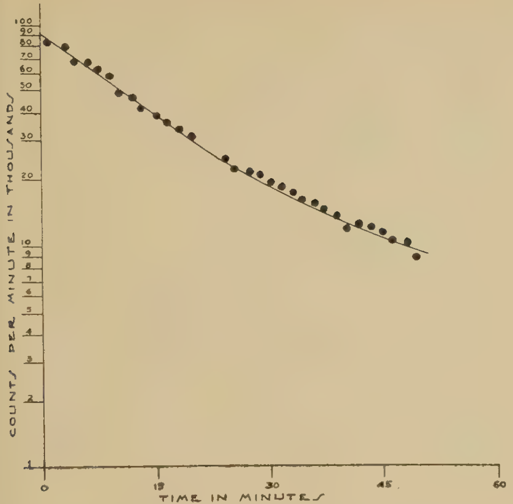


FIG. 2. Clearance of Na^{24} from subcut. tissue of rabbit. Injection of isotope was made through a 3″ long needle, which was flushed through with a small bubble of air before withdrawal.

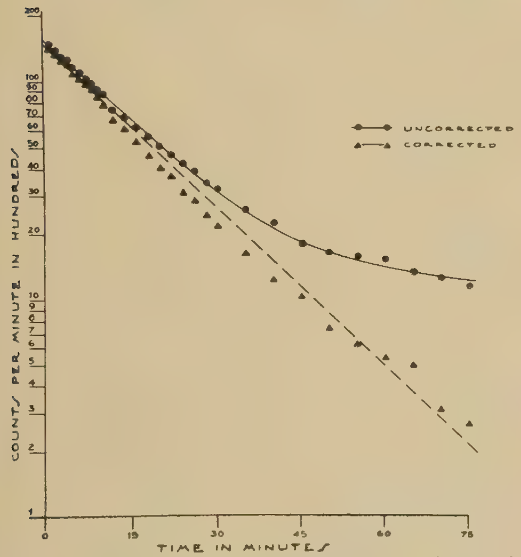


FIG. 3. Clearance of Na^{24} from subcut. tissue of cat. Solid line is the counting rate over the injection site at various times after injection. Broken line shows counts corrected by subtraction of simultaneously measured rate over control area.

the actual amount of injected isotope remaining at the injection site, and this function should show an exponential relation with time.

Results. A) Clearances obtained on animals after various intervals under anesthesia show a continuing decrease in the disappearance rate of I^{131} from the site of injection (Fig. 1). The initial rate is the most rapid with a gradual decrease throughout the period of observation. The curvilinear form of these clearance curves could not be attributed to changing blood flow unless this change continued at a uniform rate over the entire period. However, since the animals in all cases recovered from the anesthesia, and in many cases the depth of anesthesia became less while the clearance continued to fall, this explanation appears to be unlikely.

B) The results of one of the experiments outlined in section B are shown in Fig. 2. It will be noted that in spite of all precautions related to the deposition of isotope in tissue other than the subcutaneous location of the bleb, the results are still non-linear. It may therefore be argued that some other factor is involved in causing the deviation of the curve.

C. Fig. 3 presents the results of a typical experiment in which correction is made for the return of isotope to the area of the injection. It will be noted that the final corrected curve is a close approximation to a straight line, and for all practical purposes can be used as a measure of the true clearance rate.

Discussion. The results of the experiments recounted above strongly suggest that the major factor responsible for the deviation of the clearance curves from a simple exponential relationship is the return of isotope to the area under study. This factor would be of minimal importance where the isotope is highly diluted by a relatively large blood volume or where the isotope after removal from the injection site is immediately withdrawn from the blood and hence would return to the area in negligible concentration.[†] In humans, the dilution of the small amount of injected (and smaller amount of cleared) material by the relatively large extracellular fluid volume results in insignificant concentrations of isotope in the arterial blood. In the case of the small animal, the dilution is not nearly as great and the returning isotope is responsible for a significant and increasing fraction of the total activity seen by the counter over the injection site.

[†] Since iodide is rapidly removed from the circulation by the thyroid, the deviation from linearity is less marked in the iodide clearance studies than in the sodium experiments. However, the error in an iodide clearance becomes clear when the experiment is continued for a longer period of time (Fig. 1). In the shorter experiments the deviations are more dramatic when sodium is used, and hence this isotope was employed to illustrate the point in Fig. 2 and 3. It should be noted that the rate at which the thyroid removes iodide is not sufficiently great to eliminate this ion entirely from the arterial blood returning to the periphery, and thus there remains a significant deviation from linearity in these clearances.

The apparent linearity of short term experiments in animals can be accounted for if one recognizes that the arterial concentration is progressively increasing, and that while after 30 minutes the returned isotope may account for as much as half of the total activity at the injection site, during the first few minutes the correction is but a negligible one. Results based on the clearance during the first few minutes after injection are therefore admissible; the advantage to be obtained from making the correction outlined herein has to do with extending the period of observation. Several workers (*e.g.*, McGirr) have called attention to the variability in clearance during the first 3 to 5 minutes after injection of the isotope. The clearance measurements without correction must therefore be based on a relatively short length of curve beginning after the initial irregularities and ending before the returning isotope is present in sufficient concentration to cause significant error.

Conclusions and summary. We have shown that a major factor responsible for the apparently nonlinear tissue clearances as measured in small animals is the return of isotope to the area of the injection in the arterial blood. By means of a relatively simple procedure it is possible to make correction for this error and to obtain reasonable, exponential clearances. This correction is made by subtracting the simultaneously measured activity over a corresponding area of tissue from the count over the injection site.

We are pleased to acknowledge the suggestions and criticisms of Dr. M. E. Morton, S. I. Rapaport and J. Steinfeldt.

---

1. Kety, S., *Am. Heart J.*, 1949, v38, 321.

2. a. Elkin, D. C., Cooper, F. W., Jr., Rohrer, R. H., Miller, W. B., Jr., Shea, P. C., Jr., and Dennis, E. W., *Surg., Gynec., and Obst.*, 1948, v87, 1. b. Green, H. D., McFall, C. F., Pollitzer, R., and Moore, M., *Am. J. Med.*, 1950, v9, 399; c. Semple, R., McDonald, L., and Elkins, R. P., *Am. Heart J.*, 1951, v41, 803.

3. McGirr, E. M., *Clin. Sc.*, 1952, v11, 91.

4. Rapaport, S. I., Saul, A., Hyman, C., and Morton, M. E., *Circulation*, 1952, v5, 594.

5. Cooper, F. W., Jr., Elkin, D. C., Shea, P. C., Jr., and Dennis, E. W., *Surg., Gynec., and Obst.*, 1949, v88, 711.

6. Miller, H., and Wilson, G. M., *Brit. Heart J.*, 1951, v13, 227.

7. Wisham, L. H., Yalow, R. S., and Freund, A. J., *Am. Heart J.*, 1951, v41, 810.

8. a. McInally, M., Campbell, J. A., Robertson, D. J., and Douglas, D. M., *Clin. Sc.*, 1952, v11, 183. b. Cullen, M. L., *et al., Surg., Gynec. and Obst.*, 1949, v89, 722; c. Stone, P. W., and Miller, W. B., Jr., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 529.

9. Boatman, J. B., Kendrick, T. R., Franke, F. R., and Moses, C., *J. Lab. and Clin. Med.*, 1950, v36, 456.

10. Franke, F. R., Boatman, J. B., George, R., and Moses, C., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 417.

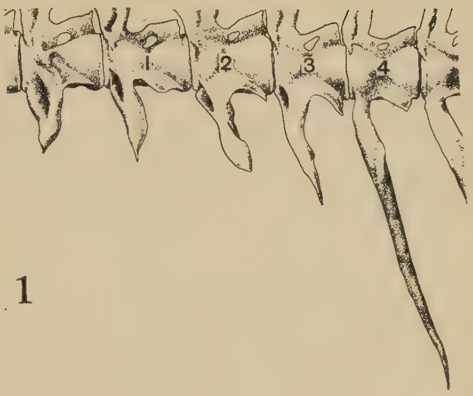
Received June 12, 1953. P.S.E.B.M., 1953, v83.

Effects of Alpha-estradiol Benzoate and Methyl Testosterone upon the Platyfish, *Xiphophorus maculatus* Skeleton.* (20446)

ALAN A. RUBIN† AND MYRON GORDON. (Introduced by H. A. Charipper)
From New York University and New York Zoological Society, New York City.

It has been shown that estrogenic hormones induce profound modifications of the pelves in pocket gophers and mice through the resorption of cartilage and bone(1,2). Recent morphological studies of 2 species of viviparous fishes indicate that there is a partial resorption of the anterior hemal spines in females(3,4) Fig. 1. In the light of these data, the present experiments were undertaken to evaluate the influence of an estrogen and an androgen upon sensitive areas of the skeleton of the common platyfish, *Xiphophorus maculatus*.

Fifty-four fish were separated into 4 groups according to age and sex. Thirty-three of them were maintained in 3-liter glass jars containing conditioned water and synthetic hormones. Once a week, the jars were thoroughly cleaned and fresh quantities of conditioned water and hormones were added. The remaining, untreated 21 fish, used as controls, were maintained under similar conditions. At the conclusion of the experiment, all of the fish were fixed in 2% KOH, stained with Alizarine Red S and mounted for study in glycerine. This technic renders the muscles transparent and stains the skeleton for immediate observation. Weekly dosages of 0.03 mg/liter of alpha-estradiol benzoate from an alcohol stock solution was placed into water jars containing platyfish males. Animals treated with this estrogen constitute Series I which contained 3 groups, A, B, C. Group A consisted of 10 immature males. Sexes were determined by genetic methods(5,6). Group B included 10 maturing males. Group C was made up of 15 adult males. Series II consisted of 19 adult females which were treated with 5.0 μg/liter of methyl testosterone.



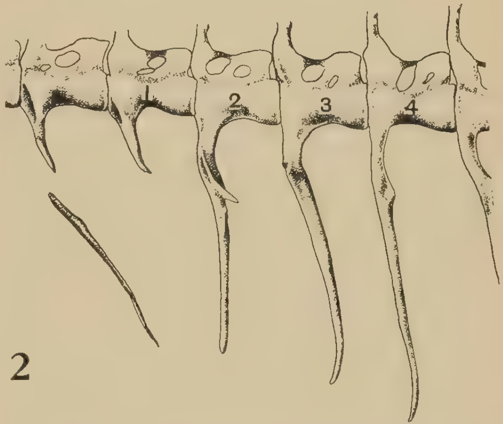


FIG. 1. A portion of the axial skeleton of a normal adult female. First 3 hemal spines have dissolved from caudal vertebrae 1, 2, 3.

FIG. 2. A portion of the axial skeleton of a normal adult male. The first hemal spine is detached from the first caudal vertebrae; succeeding hemal spines remain intact.

Results. Series I, A. Of 6 immature males treated with alpha-estradiol benzoate for 12 weeks, 5 showed dissolution effects at the anterior hemal spines. In 4 of the 5 animals, the first hemal spine of each was detached from the vertebral column and had undergone dissolution. In the fifth animal, the first 2 hemal spines were detached and were partially dissolved. All of the remaining, intact anterior hemal spines of the estrogen-treated fish were deflected posteriorly. They resembled their more posterior counterparts in structure and orientation. In the 4 control animals, on the other hand, all of the anterior hemal spines were retained and deflected anteriorly.

Series I, B. Six maturing males, 2 weeks short of sexual maturity as determined by their anal fin structure(7), were treated with estrogen for 8 weeks. In 2 fish the first hemal spine of each was dissolved and the second spine was detached from the vertebral column. The 4 remaining treated animals and all 4 controls retained their anterior hemal spines (Fig. 2).

Series I, C. The anterior hemal spines of 9 adult males, treated with estrogen for 8 weeks, were indistinguishable from those of 6 controls (Fig. 2).

Series II. Two series of small, sexually dimorphic bones called mesonosts and baseosts,

---

* Abridgement of part of thesis submitted by Alan A. Rubin in partial fulfillment of the requirements for the degree of Master of Science, Graduate School of Arts and Sciences, New York University. Supported in part by a grant from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

† Present address: Schering Corp., Bloomfield, N. J.

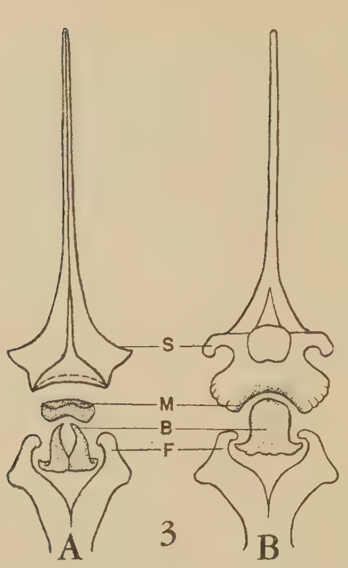


FIG. 3. Anterior view of a unit of the anal fin suspensorium at the level of the fifth interhemal spine (S). A. Normal adult female: the mesonost (M) and baseost (B) are discrete, the baseost being bipartite. B. Normal adult male: the mesonost (M) is fused to the base of the interhemal spine (S) and the baseost (B) is unipartite. Abbreviations: S—interhemal spine; M—mesonost; B—baseost; F—anal fin ray.

comprise the basal portion of the anal fin suspensorium. In normal adult females they are discrete; the baseosts being bipartite (Fig. 3A). In normal adult males, the mesonosts are apparently missing, but they are actually fused to the ventral surfaces of the interhemal spines (Fig. 3B). The baseosts in males are discrete but unipartite.

In 6 of the 12 adult females treated with methyl testosterone for 8 weeks, the entire series of mesonosts was fused to the ventral surfaces of the first 8 interhemal spines. In the 6 remaining animals, the anterior mesonosts were fused while the posterior ones were close to but not fused to their corresponding interhemal spines. In all of the treated animals, the baseosts had begun to take on the unipartite condition characteristic of normal males. The 7 control females had normal, discrete mesonosts and bipartite baseosts.

Summary. Alpha-estradiol benzoate induced the dissolution of one or two anterior hemal spines in a significant number of immature and maturing male platyfish but elicited no response in adult males. Methyl testosterone, administered to adult female platyfish, induced the fusion of 2 series of small, basal bony elements, the mesonosts and baseosts. The mesonosts ordinarily discrete, became fused to the ventral surfaces of the interhemal spines. The baseosts, normally bipartite, became unipartite.

---

1. Hisaw, F. L., *J. Exp. Zool.*, 1925, v42, 411.
2. Gardner, W. U., *Am. J. Anat.*, 1936, v59, 459.
3. Vivien, J., and Mohsen, T., *Compt. Rend. Soc. Biol.*, 1951, v145, 1364.
4. Rubin, A. A., Thesis, 1953, Library, New York University.
5. Gordon, M., *Genetics*, 1927, v12, 253.
6. ———, *Genetics*, 1947, v32, 8.
7. Grobstein, C., *Univ. Calif. Pub. Zool.*, 1940, v47, 1.

Received June 15, 1953. P.S.E.B.M., 1953, v83.

Rate of Disappearance and Metabolism of Hydrocortisone and Cortisone in the Synovial Cavity in Rheumatoid Arthritis.*† (20447)

HILDEGARD WILSON, JOHN GLYN, EDWARD SCULL, CURRIER MCEWEN, AND MORRIS ZIFF.

From the Departments of Chemistry and Medicine, and the Study Group on Rheumatic Diseases, New York University-Bellevue Medical Center, New York City.

Several investigators have found that the acetate of hydrocortisone (Kendall's Compound F, 11-hydroxy-corticosterone) is effective in suppressing inflammation when injected into joints with synovial effusions(1-3). Since

---

* This investigation was supported by grants from the Masonic Foundation for Medical Research and Human Welfare, and the American Cancer Society.

† The steroid hormones used in these experiments were generously supplied by Dr. Elmer Alpert, of Merck & Co.

the synovial fluid is easily accessible to analysis, this observation affords an opportunity of studying some of the processes which may accompany the therapeutic response in the arthritic joint.

Cortisone acetate has been found to be far less active than hydrocortisone acetate when administered intra-articularly, while both are known to relieve rheumatoid arthritis when administered systemically. It was of interest, therefore, to determine whether difference in their rates of absorption from the synovial cavity would account for the difference in activity. Accordingly quantitative assays have been performed on extracts of synovial fluid aspirated at various time intervals after the injection of cortisone, hydrocortisone and their acetates.

Another explanation for the difference in local effectiveness might be that cortisone must be converted to hydrocortisone in order to become therapeutically active, and that this transformation cannot adequately be effected by synovial tissue, though it does presumably occur elsewhere in the body when cortisone is given systemically. It is also possible, finally, that both compounds must be converted to an active substance, as yet unknown, before exerting their effect at the site of inflammation. A search for metabolites of cortisone, hydrocortisone and their acetates in the synovial fluid was, therefore, undertaken.

Experimental. Injection of compounds and aspiration of fluid. The 6 subjects were patients with rheumatoid arthritis who had effusions in the knee joints. They were receiving maintenance cortisone therapy in a dosage of 75 to 100 mg of cortisone acetate per day orally. In most experiments a control sample of synovial fluid was withdrawn before injection of steroid. If a subject had effusions in both knees, the entire fluid from one knee was used as control. If only one effusion was present, approximately half the fluid was withdrawn for control analyses before injection of steroids. In several instances a 2-week interval was allowed after aspiration of all the fluid for control analyses, during which the fluid reaccumulated, and the injections were then performed.

Cortisone, hydrocortisone, and their acetates, in quantities of 25 to 100 mg were injected into the knees in saline suspension.‡ The knees were flexed and massaged repeatedly to effect mixing in the synovial fluid, and after various time intervals all the fluid was withdrawn. Two 20 ml portions of physiological saline solution were then injected, and withdrawn after further flexion and massage. After noting the volumes, the original fluid was combined with the saline washes. In a number of experiments it was possible repeatedly to recover 20 ml volumes of injected physiological saline completely, and it is believed, therefore, that the synovial fluid containing the injected compounds as well as the saline washes were also accessible to complete removal.

Model experiments were performed to test whether simple settling out to an inaccessible region of the synovial cavity would be an appreciable factor in recovery experiments. Twenty-five ml of synovial fluid aspirated from the knee joint of a patient with rheumatoid arthritis was placed in a cellophane dialyzing bag. One ml (25 mg) of cortisone acetate suspension was pipetted into the center of this fluid. The suspended steroid immediately rose to the top of the fluid. The bag was tied off just above the upper fluid level, then alternately squeezed, released, and bent, in simulation of flexing and massage of the knee joint. After one hour, approximately 5 to 10% of the steroid material had settled to the bottom, leaving most of the steroid suspended.

Extraction. The combined synovial fluid and saline washes were diluted with nine volumes of distilled water, shaken to break up the viscous masses of mucoid material, then extracted three times in separatory funnels, with a volume of chloroform equal to one-third the volume of the diluted fluid. After each extraction the resulting emulsion was centrifuged, and the aqueous layer obtained was recombined with the main portion. The combined chloroform extracts were washed once with cold 5% sodium carbonate solution and 3 times with distilled water. The chloroform

---

‡ This was the saline diluent provided by Merck and Co. which contains suspending agents and 0.9% benzyl alcohol.

TABLE I. Recoveries of Cortisone, Hydrocortisone, and Their Acetates from Synovial Fluid and Water.*
Procedure controls

			Vol extracted, ml		% recovered by assays†		
Exp. No.	Compound added	mg added	Water	Synovial fluid	FS	MRS	PS‡
1	E free	25.0	250	0	81	75	86
2	”	.5	250	0	86	70	67
3	F ac	25.0	225	25	§	—	68
4	”	”	”	”	§	—	63
5	E free	”	”	”	86	68	63
6	F free	”	”	”	93	64	65
				Avg	89	66	65
7	E ac	.5	225	25	§	—	54
8	F ac	”	”	”	§	—	60
9	E free	”	”	”	106	133	90
10	F free	”	”	”	68	52	27

* E is cortisone; F is hydrocortisone; E ac and F ac are their acetates.

† FS, formaldehydogenic steroids; MRS, molybdate reducing steroids; PS, Porter-Silber reacting steroids.

‡ Calculated in equivalents of the compound listed as a reference standard.

§ The acetates do not react in the formaldehydogenic assay.

was then evaporated to dryness at temperatures not over 40°C, *in vacuo,* and the residue quantitatively transferred to a small vial.

Quantitative assays. 1) Porter-Silber. All extracts were assayed by a micro modification of the Porter-Silber reaction(4) using phenylhydrazine in sulfuric acid. The appropriate standard was used if the compound to be assayed was known. Unknown mixtures are expressed as mg cortisone equivalent.

2) Periodic acid oxidation. The formaldehyde liberated by periodic acid was determined by a micro modification of the direct reaction of Lowenstein, Corcoran and Page (5).

3) Molybdate reduction. A micro adaptation of the acid molybdate reduction assay of Heard and Sobel(6) was employed for some extracts.

Paper chromatography. The procedure of Burton and Zaffaroni(7,8) was employed without major modifications. The steroids were located by cutting 2 mm wide test strips from the main chromatogram, and applying in turn the triphenyl tetrazolium chloride reduction test(7), the osmium tetraoxide reduction test (9), and by noting the areas of fluorescence after application of concentrated sulfuric acid (8). The chromatograms were developed in toluene-propylene glycol (diluted 1:1 with methanol) for periods varying from 5 to 9 days, in order to separate slow moving compounds, after which the runoffs were reapplied and developed for shorter periods in order to yield the faster moving compounds.

Results. 1. *Procedure controls.* Control recovery experiments were performed in order to check the precision of the method. Table I shows that when 25 mg of cortisone alcohol were added to 250 ml of water, and put through the entire extraction and assay procedure, an average of 81% was recovered by the 3 assay methods. When the 4 steroids were added to 25 ml of human synovial fluid, which was then diluted with 225 ml of water, the recoveries were somewhat lower by the reduction and Porter-Silber methods. When 0.5 mg quantities were added, the values showed greater deviations from the mean assay.

2) *Post injection recovery experiments.* Table II summarizes the assay findings on synovial fluids withdrawn at various intervals after injection of cortisone and hydrocortisone acetates and free alcohols. Values in the 3 right hand columns have been corrected for the percentage recoveries obtained in the procedure control experiments. When the fluid was removed only 5 minutes after injection of 50 mg of steroid, there was already an average diminution of about 40% in the corrected assay. One hour after the injection of

TABLE II. Recoveries of Hydrocortisone, Cortisone, and Their Acetates* after Injection into Synovial Cavity.

Compound injected	Patient	mg injected	Interval before aspiration (min.)	% recovery by assays†					
				Uncorrected			Corrected for procedure controls‡		
				FS	MRS	PS	FS	MRS	PS§
E ac	G. R.	50	5	—	—	30	—	—	46
F free	G. R.	50	5	35	—	56	39	—	86
F ac	E. S.	50	60	—	—	6	—	—	9
”	M. F.	50	60	—	—	9	—	—	14
”	G. R.	25	60	—	—	14	—	—	21
E ac	G. R.	50	60	—	—	8	—	—	12
”	H. G.	100	120	—	—	13	—	—	20
”	R. M.	50	480	—	—	<1	—	—	<1
F free	G. R.	100	30	16.1	14.2	18.3	18.1	21.5	28.2
”	G. R.	100	30	18.0	18.9	10.5	20.2	28.7	16.2
”	G. R.	100	30	7.9	9.9	10.2	8.9	15.0	15.7
”	E. W.	100	30	21.3	16.5	18.1	23.9	25.0	27.8
			Avg	15.8	14.9	14.3	17.8	22.6	22.0
F free	N. B.	100	180	1.1	2.7	5.0	1.2	4.0	7.7
”	G. R.	100	180	.3	1.4	1.1	.3	2.1	1.7
”	G. R.	100	180	.6	1.1	1.2	.7	1.7	1.8
”	G. R.	100	180	.3	.8	.4	.3	1.2	.6
			Avg	.6	1.5	1.9	.6	2.3	3.0
E free	L. M.	100	180	3.4	7.1	1.6	3.8	10.7	2.5
”	G. R.	100	180	2.5	4.6	1.2	2.8	7.0	1.8
”	G. R.	100	180	.8	5.0	.5	.9	7.6	.8
”	G. R.	100	180	.8	3.4	1.0	.9	5.1	5.2
			Avg	1.9	5.5	1.1	2.1	7.6	2.6

* E is cortisone; F is hydrocortisone; E ac and F ac are their acetates.

† FS, formaldehydogenic steroids; MRS, molybdate reducing steroids; PS, Porter-Silber reacting steroids.

‡ Each assay is corrected for the avg % recovery of exp. 3, 4, 5 and 6 shown in Table I.

§ Calculated in equivalents of the injected compound as a reference standard.

hydrocortisone acetate and cortisone acetate the steroid remaining had decreased to an average of 14% of that injected. In four identical experiments 30 minutes after the injection of 100 mg hydrocortisone free alcohol, the average recovery was 21%. Similar experiments 3 hours after injecting 100 mg of either hydrocortisone or cortisone free alcohols gave an average recovery of only about 2%.

3. *Paper chromatographic analyses.* A series of extracts prepared from synovial fluid samples of a single subject (G. R.), after the injection of hydrocortisone free alcohol, were submitted to chromatographic analysis on paper. On each occasion 50 mg of the steroid were injected, and the entire synovial fluid subsequently withdrawn and combined with saline washings. The results are depicted in Fig. 1. Porter-Silber assays on the crude extracts showed that 29.2 mg or only 56% of the injected steroid could be recovered from the synovial fluid 5 minutes after injection. In the half-hour and one hour experiments the assays were 4.4 mg and 0 mg respectively, again showing the rapid disappearance of the injected steroid.

The left hand chromatogram in Fig. 1 shows the approximate position of known pure compounds run on parallel strips when 100 μg were applied on a one cm paper and tested by the reduction spot tests. The second chromatogram of Fig. 1 shows the results with the combined pre-injection control fluid extracts, run on one cm papers. Since this patient had been receiving 100 mg of cortisone acetate orally per day for a period of 18 months, it is interesting to note that no cortisone (compound E) could be seen on the control fluid chromatogram. Only one faint band was seen, indicating the presence of about 50 μg of a substance having a mobility similar to that of hydrocortisone (compound F). The third chro-

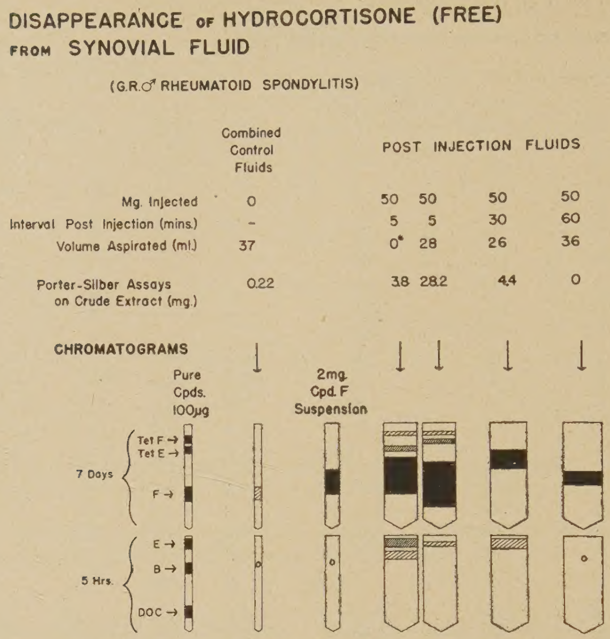


FIG. 1. Disappearance of hydrocortisone (free) from synovial fluid. "Tet F" is tetrahydro-hydrocortisone; "Tet E" is tetrahydrocortisone; "F" is hydrocortisone; "E" is cortisone; "B" is corticosterone; "DOC" is 11-desoxycorticosterone. Although tetrahydro-hydrocortisone was not available as a pure reference standard, its mobility is believed to be as shown on the chart, by analogy both with isomeric adrenal cortical compounds, and with chromatographic fractions of urine extract shown to contain this compound.

* No effusion was present in the joint when this experiment was done. The steroid recovered was obtained by washing the joint with two 20 ml portions of saline solution.

matogram shows that no compounds other than hydrocortisone were detected in the suspension used for injection.

The 4 post-injection chromatograms, run on 5 cm papers, revealed chiefly unchanged hydrocortisone. Additional weak reducing bands indicated metabolic products of hydrocortisone retaining the α-ketol structure. In order to concentrate these materials, all sections of each of the 4 papers above the hydrocortisone band were eluted and combined. Eluates of the four 5-hour papers containing the faster moving compounds were also combined.

Fig. 2 shows the results obtained when the eluates of these combined cuts were rechromatographed on narrower strips. Three distinct bands were found. The slow moving material found above the hydrocortisone band yielded a band which had scarcely moved after 7 days. The combined eluates of the previous 5-hour papers yielded 2 more bands, having mobilities similar to cortisone and corticosterone (compound B) respectively.

In Fig. 3 are shown the results of experiments in which cortisone acetate was injected intra-articularly. The extract of 130 ml of control fluid obtained from this subject (H. G.) was chromatographed on a 0.5 cm paper. Only one faint, slow moving band was seen. Chromatography of the injected suspension revealed the presence of free cortisone as well as the acetate. In the post-injection experiment, the synovial fluid was aspirated 2 hours after the injection of 100 mg of cortisone acetate. After extraction and chromatography on a one cm paper for 5 days, a strong band was seen above the free cortisone. The mobility of this material was similar to that of tetrahydrocortisone. The sulfuric acid chromogen absorption curve (10) of this band was similar to that of tetrahydro cortisone and definitely indicated the absence of hydrocortisone in this band. On reapplying the runoff from the 5-day paper for a period of 5 hours, 2 weak bands moving faster than the cortisone acetate were seen.

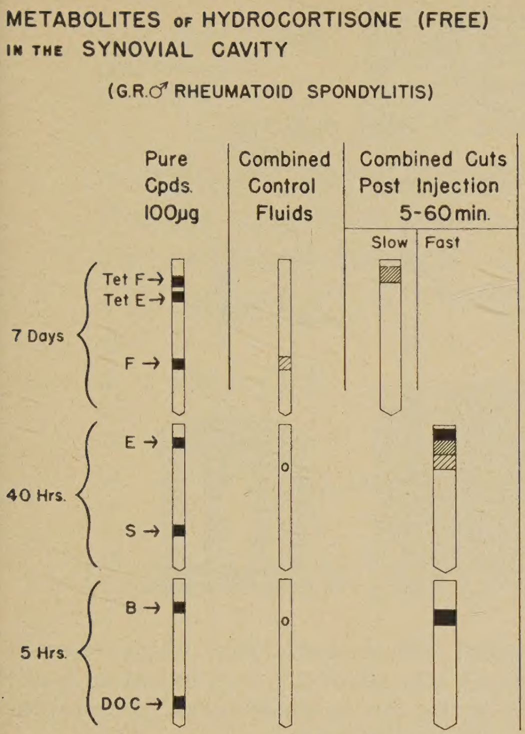

METABOLITES OF HYDROCORTISONE (FREE) IN THE SYNOVIAL CAVITY
(G.R.♂ RHEUMATOID SPONDYLITIS)
Pure Cpds. 100µg
Combined Control Fluids
Combined Cuts Post Injection 5-60 min.
Slow
Fast
7 Days
Tet F →
Tet E →
F →
40 Hrs.
E →
S →
5 Hrs.
B →
DOC →
o
o

FIG. 2. Metabolites of hydrocortisone (free) in the synovial cavity. "Tet F" is tetrahydro-hydrocortisone; "Tet E" is tetra-hydrocortisone; "F" is hydrocortisone; "E" is cortisone; "S" is 11-desoxy-17-hydroxy corticosterone; "B" is corticosterone; "DOC" is 11-desoxycorticosterone.

Five minute and one hour experiments (Fig. 3) were performed on another subject (G. R.), after the injection of 50 mg of cortisone acetate. When these extracts were chromatographed, no new bands were seen after 5 minutes in the knee, while material moving more slowly than cortisone was seen after one hour.

Discussion. The apparent rapid disappearance of the relatively insoluble corticosteroids, cortisone and hydrocortisone, from the synovial fluid was quite surprising. It is possible that the low recoveries after injection are due in part to the mechanical inaccessibility of a portion of the injected material. When cortisone acetate suspension was injected into synovial fluid contained in a cellophane bag, however, mechanical settling occurred too slowly to account for the extremely low recoveries after one and 3 hours. It seems likely, therefore, that a definite adsorption or absorption process involving the synovial tissue takes part in the removal of injected steroid from the synovial fluid. Since, as indicated by our results, hydrocortisone and cortisone are removed from the synovial fluid at essentially the same rate, the difference in their therapeutic activity locally cannot be ascribed to a difference in ease of absorption by the synovial membrane.

Chromatographic analysis of the post-injection extracts indicates the presence of metabolites of the compounds originally injected. Conclusive identification of these metabolites must await the accumulation of sufficient material to yield more positive information than that afforded by spot tests on the paper chromatograms. These experiments are now in progress. The appearance of well localized bands which were not seen in either the control synovial fluid or in the suspension which was injected, are taken as strong evidence, however, that chemical transformation of the steroid compounds has been effected either by the synovial tissue, or by the inflammatory cells infiltrating the synovial membrane as a result of the arthritic process. It is believed probable that the metabolites found in synovial fluid represent the back diffusion of a small proportion of the new compounds being formed.

The finding of metabolites, in this instance, implies that the target tissue has metabolized the hormone which is acting upon it. On the basis of the present data it cannot, of course, be decided whether or not such transformations are essential to the therapeutic activity of the hormone. There is some suggestion, however, that hydrocortisone and cortisone are metabolized to different products, at least in part. The strongest bands in the hydrocortisone experiments were faster moving than hydrocortisone, whereas after cortisone injection the most abundant material had a slow mobility similar to that of tetrahydrocortisone.

Proof of intra-articular metabolism of steroid compounds requires elimination of the possibility that the metabolites which were observed had actually been formed elsewhere, as in the liver, for instance, and had returned to the synovial fluid from the general circulation, following absorption of the injected com-

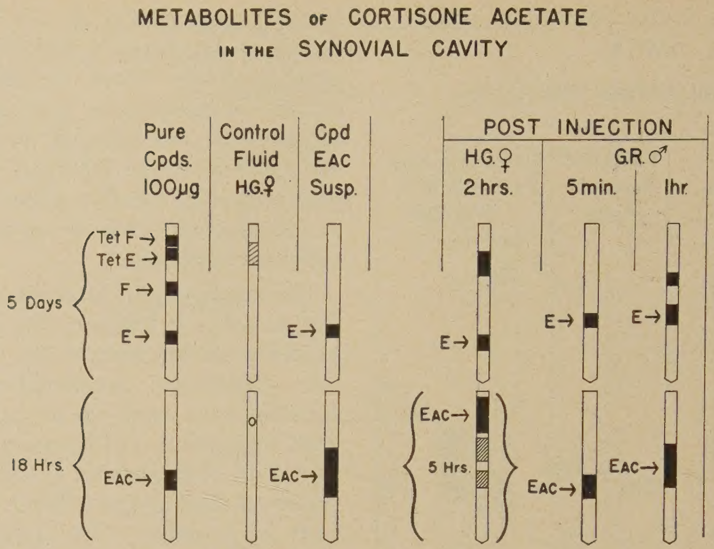


FIG. 3. Metabolites of cortisone acetate in the synovial cavity. "Tet F" is tetrahydro-hydrocortisone; "Tet E" is tetrahydrocortisone; "E" is cortisone; "E_{AC}" is cortisone acetate.

pound from the joint. This possibility was investigated in an experiment in which 50 mg of hydrocortisone acetate were injected into one knee (Subject G. R.). Six hours later this knee was aspirated and fluid also removed at the same time from the contralateral joint. The chromatograms of fluid from the contralateral joint gave no evidence of any steroid whatsoever, while the fluid from the injected knee showed three definite bands in addition to the unchanged hydrocortisone acetate.

Further experiments now under way are directed toward more definite characterization and comparison of the intra-articular metabolites of hydrocortisone in both rheumatoid arthritis and other arthritides.

Summary. Quantitative assays have been performed on extracts of synovial fluid aspirated from patients with rheumatoid arthritis, following intra-articular injection of cortisone, hydrocortisone, and their acetates. After one hour and 3 hours about 85% and 98% respectively had disappeared. There was no marked difference in rate of disappearance among the 4 compounds. Paper chromatographic analyses of post-injection extracts revealed well localized bands which were not seen in the control fluid from uninjected joints, indicating that metabolites were formed within the synovial cavity.

---

1. Ziff, M., Scull, E., Ford, D., McEwen, C., and Bunim, J. J., *A. M. A. Arch. Int. Med.*, 1952, v90, 774.

2. Hollander, J. L., Brown, E. M., Jr., Jessar, R. A., and Brown, C. Y., *J.A.M.A.*, 1951, v147, 1629.

3. Stevenson, C. R., Zuckner, J., and Freyberg, R. H., *Ann. Rheum. Dis.*, 1952, v11, 112.

4. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*, 1950, v185, 201.

5. Lowenstein, B. E., Corcoran, A. C., and Page, I. H., *Endocrinol.*, 1946, v39, 82.

6. Heard, R. D. H., and Sobel, H., *J. Biol. Chem.*, 1946, v165, 687.

7. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.*, 1951, v188, 763.

8. Zaffaroni, A., and Burton, R. B., *J. Biol. Chem.*, 1951, v193, 749.

9. Manaro, J. M., and Zygmuntowicz, A., *Endocrinol.*, 1951, v48, 114.

10. Zaffaroni, A., *J. Am. Chem. Soc.*, 1950, v72, 3828.

Received June 25, 1953. P.S.E.B.M., 1953, v83.